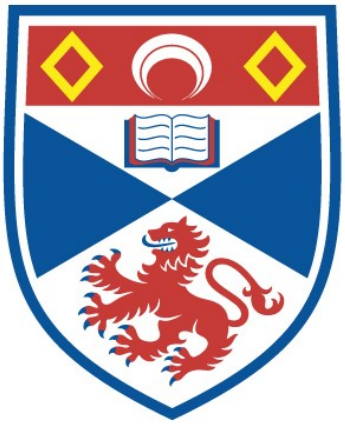
THE EFFECTS OF RHEUMATOID ARTHRITIS AND AGE ON SOME PROPERTIES OF HUMAN TENDON COLLAGEN

David M. Worsnip

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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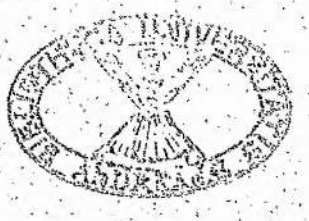
By

DAVID M. WORSNIP

A thesis presented to the University of St. Andrews for the Degree of Doctor of Philosophy.



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D E C L A R A T I O N

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry in the United College of St. Salvator and St. Leonard, St. Andrews, under the direction of Professor G.R. Tristram.

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C E R T I F I C A T E

I hereby certify that David H. Worsnip has spent nine terms engaged in research work under my direction and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

A C A D E M I C R E C O R D

I matriculated at the University of St. Andrews in October 1958, and graduated with the degree of Bachelor of Science, Second Class Honours in Biochemistry, in June 1962. My subsidiary subjects were: Zoology, Chemistry, and Physics, at General Level, and Chemistry at Special Level.

In October 1962 I matriculated as a research student in the Department of Biochemistry, St. Andrews University.

In October 1964, I accepted the post of Assistant Lecturer in the Department of Biochemistry.

A C K N O W L E D G E M E N T

I should like to thank Professor G.R. Tristram for helpful discussion and criticism during this research.

Financial support from The Medical Research Council during the first two years of this research, in the form of "A Scholarship For Training in Research Methods", is gratefully acknowledged.

I should also like to thank all those who helped to maintain a steady supply of normal and rheumatoid tissue from Glasgow Royal Infirmary, Craigtoun Maternity Hospital, St. Andrews, and Princess Margaret Rose Orthopaedic Hospital, Edinburgh.

Thanks are also due to Mrs. A. Serafini-Fracassini for performing the amino acid analyses, and to Mr. W.J. Blyth for the photography.

Finally, thanks are due to my wife for her help and encouragement during the preparation of this thesis.

C O N T E N T S

If by some magic solution one could dissolve all the connective tissue of the body, all that would remain would be a mass of slimy epithelium quivering muscle and frustrated nerve cells.

Arcadi.

I N T R O D U C T I O N

CONNECTIVE TISSUE AND ITS COMPONENTS.

Bichat first distinguished a fibrous tissue when postulating his basic idea that there were a limited number of tissues from which organs were formed. The whole concept of connective tissue has risen from this observation, the term "bindegewebe" being introduced around 1830 by the German histologist Johann Müller.

Virchow's theory of cellular activity meant that the functional significance of connective tissue went unheeded almost until the beginning of the last decade when a renewal of interest occurred due to rapid advances in molecular biology and the classic concept of Klemperer and his group (Klemperer, Pollack and Baehr, 1942) of a series of connective tissue disorders. This period has been called 'the connective tissue renaissance' by Robb-Smith (1954).

The connective and skeletal tissues are concerned with the formation and maintainance of structure throughout the body. They have as a common origin the embryonic mesenchymal cell which in the course of differentiation forms the connective tissue proper, cartilage and bone.

It is convenient to classify the extracellular moiety into the following phases. (Fitton-Jackson (1961)).

1. The fibrous proteins - collagen, elastin and reticulin.
2. The acid mucopolysaccharides, usually found as complexes with noncollagenous protein.
3. The neutral or heteropolysaccharides.
4. The interstitial fluids and their content of plasma proteins.
5. Lipid constituents.

It is the purpose of this thesis to consider the effects of age and disease, in particular rheumatoid arthritis, on some properties of the collagen component of the fibrous phase of connective tissue.

THE COLLAGEN COMPONENT OF CONNECTIVE TISSUE.

Collagen is involved in almost every aspect of the construction of the vertebrate body and makes up the major fibrous element of extracellular connective tissues. It occurs in almost all types of multicellular animals, invertebrates and vertebrates. It exists in various forms in vivo; as the almost pure protein in fine threads present in the tail tendon of rats and in the tendons of humans; as a network of fine fibres embedded in a ground substance of mucopolysaccharid in the various forms of cartilage; and in certain specialised conditions, such as the cornea where layers of aligned collagen fibres are superimposed so that the fibre directions in alternate layers are at right angles, in the vitreous where an open mesh of collagen fibres retains the "vitreous" hyaluronic acid gel and in bone where collagen forms the gel phase in which the solid mineral component develops.

Neuberger and Richards (1964) estimated the collagen content of mammals to be about 20-25% of the total protein, Harkness, Harkness and James (1958) gave a figure of 20% for the tissues of the mouse. These figures were somewhat lower than previous estimates of 30-35%.

EARLY WORK ON THE CHEMISTRY OF COLLAGEN.

Up to the turn of the century collagen had been recognised by histologists as the protein which made up bundles of fibres constituting the bulk of the solid matter of the skin corium and which was converted to gelatin or glue on boiling with water. An elementary analysis of collagen was published by Hofmeister as early as 1878 and he then advanced the idea that collagen was an anhydride of gelatin, the two forms being interconvertible. At the turn of the century the idea that proteins were built up entirely, or almost entirely, of amino acids was just beginning to be accepted, although it had first been proposed in 1873 by Hlasiwetz and Habermann after work on casein. The idea that amino acids were linked together by the "peptide bond" had just been put forward by Fischer and Hofmeister. Fourteen of the eighteen amino acids known now to be present in collagen had been isolated although not all had been characterised structurally. The characteristic amino acid of collagen, "hydroxyproline", was isolated from a gelatin hydrolysate by Fischer in 1902. Glycine in fact had also been isolated from gelatin, but many years previously. Gelatin proved to be a useful and readily available material for many of these preliminary investigations of protein structure.

The first amino acid composition of gelatin was published by Plimmer in 1912 using Fischer's (1901) ester distillation method and this was followed by a second set of

results published in 1920 by Dakin using a butanol extraction method. It was not until 1948 that Bowes and Kenten finally published a set of values which was generally accepted to represent the true state of affairs, accounting as it did for over 99% of the nitrogen of collagen, (TABLE 1).

TABLE 1.

Amino acid Analyses of Gelatin & Collagen.

	Plimmer (1912) Gelatin	Dakin (1920) Gelatin	Bowes & Kenten (1948) Collagen
Glycine	16.5	25.5	26.2
Alanine	0.8	8.7	9.5
Valine	1.0	0.0	3.4
Leucine Isoleucine	2.1	7.1	5.6
Phenylalanine	0.4	1.4	4.2
Tyrosine	0.0	0.01	1.4
Serine	0.4	0.4	3.4
Methionine	-	-	0.8
Threonine	-	-	2.4
Proline	7.7	9.5	15.1
Hydroxyproline	3.0	14.1	14.0
Aspartic Acid	0.6	3.4	6.3
Glutamic Acid	0.9	5.8	11.3
Arginine	7.6	8.2	8.8
Histidine	0.4	0.9	0.8
Lysine	2.8	5.9	4.5
Hydroxylysine	-	-	1.3
TOTAL	44.2	90.9	119.0

All results expressed as g. amino acid per 100 g. Protein.

THE AMINO ACID COMPOSITION OF COLLAGEN.

From the overall amino acid composition of collagen it is clear that its most characteristic feature is the unusual nature of this composition, See Table 2. Mammalian collagens are rich in glycine and in all collagens so far investigated this amino acid constitutes about one third of the total residues. There is also present an unusually high content of the imino acids, proline and hydroxyproline, the hydroxyproline content being so characteristic as to be necessarily present before a protein is classed as a collagen. The structural significance of the imino acids will be discussed at a later stage. Small quantities of tyrosine, histidine and the sulphur containing amino acids occur but only tyrosine may have any significance with regard to structural effects. Cystine occurs only occasionally and then only in trace amounts (Tristram and Smith, 1963). Hydroxylysine is the second constituent of the collagens which is necessary for a protein to be classed in this group. It has recently been suggested that it has some significance in the binding of carbohydrates (Butler and Cunningham, 1965, 1966). Tryptophan does not occur in the majority of collagens.

Mammalian collagens appear to show small variations between species and, although many of those differences may be within the limits of error of the methods used, there is good evidence that there are definite differences in collagens from

TABLE 2

The Amino Acid Composition of Several Collagens

	Carp Swim Bladder [1]	Dog Fish Skin [2]	Rat Skin [1]	Calf Skin [3]	Human Skin [4]	Rat Tail Tendon [1]	Human Tendon [5]
Glycine	333	339	331	320	330	331	324
Alanine	125	110	106	112	110	107	111
Valine	17.6	27.8	24.0	20.0	24.4	22.9	25.4
Leucine	20.1	25.5	23.8	25.0	24.3	23.6	26.0
Isoleucine	9.8	16.7	10.8	11.0	9.5	9.6	11.1
Phenylalanine	13.4	12.4	11.3	13.0	12.0	11.9	14.2
Tyrosine	3.4	2.7	2.4	2.6	2.8	3.9	3.6
Serine	37.0	59.0	43.0	36.0	35.6	43.0	36.0
Methionine	16.0	15.7	7.8	4.3	6.2	8.4	5.7
Threonine	27.6	24.0	19.6	18.0	17.5	19.9	18.5
Proline	116	99.0	121	138	128	122	126
Hydroxyproline	76.5	60.5	93.0	94.0	94.1	94.2	92.1
Aspartic Acid	47.0	44.0	46.0	45.0	45.0	45.0	48.4
Glutamic Acid	71.0	69.0	71.0	72.0	73.0	71.0	72.3
Arginine	52.0	53.0	51.0	50.0	51.0	50.0	49.0
Histidine	3.6	11.7	4.9	5.0	4.8	4.1	5.4
Lysine	25.6	25.7	28.1	27.0	26.9	26.9	21.6
Hydroxylysine	7.0	6.3	5.7	7.4	5.8	6.6	8.9
Amide N.	(40.0)	(38.0)	(41.0)	-	(36.9)	(40.0)	-

Values: Residues per 1000 Total Residues.

1 Piez Eigner and Lewis (1963). 2 Lewis and Piez (1964). 3 Piez and Gross (1960)
4 Bornstein and Piez (1964). 5 Eastoe (1955).

different mammalian species (Bantoe and Leach, 1958). These differences were brought to light in the studies of complement fixation by Watson, Rothbard and Yanamee (1954), where fixation was specific with respect to species, but not to the tissue source of the collagen within a species. Piez and Likins (1957), have found relatively large differences in the hydroxylysine content of collagens isolated from tissues such as bone, tail tendon and dentine of the rat.

The invertebrate and fish collagens are of a similar nature and have the general characteristics of the mammalian collagens but, in general, the content of any one amino acid, apart from glycine is much more variable (Piez and Gross, 1959 & 1960). The significance of the variability of the proline and hydroxyproline content has been discussed by Watson (1958), and Piez and Gross (1959 & 1960). The content of cystine is higher in the invertebrate collagens and recently McBride and Harrington (1965), have reported 27 half residues of cystine per 1,000 total residues of amino acids in Ascaris cuticle collagen, and suggested that disulphide linkages constitute cross links in this type of collagen.

Carbohydrate associated with Collagen

Consden (1953), Gross (1957) and Oneson and Zacharias (1960), were of the opinion that collagen was associated with a small amount of carbohydrate, mostly hexose (about 0.7%) and a small amount of hexosamine. Bowes, Elliott and Moss (1955) and Moss (1955), were of the opinion that the carbohydrate moiety was an impurity. However, studies by Grassmann and his colleagues (see Grassmann, Hofmann, Kuhn, Hörmann, Endres and Wolf 1957a) have shown that the hexosamine was not an essential part of the procollagen fibril as it could be removed completely this has also been shown to be the case in salt soluble collagen by Fessler (1960) and Jackson, Leach and Jacobs (1958). However the hexose which remains firmly attached to the collagen even after a mild periodate oxidation (Hörmann and Fries 1958), about 0.5%, was suggested to be an integral part of the collagen fibril by Grassmann et al., (1957a), possibly bound to collagen by O-glycosidic linkages (Grassmann, Hörmann and Hafter, 1957c).

Oneson and Zacharias (1960), isolated an oligosaccharide from a partial hydrolysate and suggested that carbohydrates may be linked to the molecule in the form of a polysaccharide.

At the present time a certain amount of hexose is believed to be involved in the ester cross links in collagen (Gallop 1964; Hörmann 1960a & 1962); and glucose and galactose are believed to be linked as a disaccharide to the hydroxyl

group of hydroxylysine (Butler and Cunningham, 1965 & 1966). These suggestions will be mentioned in more detail in a later section.

Blumenfeld, Paz, Gallop and Seifter (1963) have indicated that there were five residues of glucose and seven of galactose per tropocollagen molecule (Ichthyocol, molecular weight 320,000). These residues were further found to be bound via carbon 1 by an O-glycosidic link. More recently Francois and Glimcher (1966), have demonstrated that the single α components of cod fish skin collagen contain hexose as an integral part of their structure; (α_1 had 1.9 moles/1000 moles amino acids; α_2, 0.9 moles/1000; α_3, 1.7 moles/1000). They further showed a content of 11.2 moles hexose per mole of acid soluble collagen, a figure close to that quoted by Blumenfeld et al., (1963). They suggested that the discrepancy between total subunit content and collagen content may be due to the presence of non-collagenous, hexose containing protein.

The Primary Structure of Collagen.

Gustavson (1946) stated that, from evidence thus far obtained, it was apparent that a certain periodicity in the arrangement of amino acid residues was indicated for proteins in general and in particular for the fibrous proteins. It was thought, at this time, that the amino acid sequence of collagen (determined on gelatin) conformed to the general structure G-P-X-G-P-X-G-P-X, G being glycine, P being proline or hydroxyproline and X the remainder of the amino acids. This hypothesis was suggested by Bergmann (1935, 1939) and Bergmann and Niemann (1936, 1937), and supported by Grassmann's (1936) investigations as well as studies using X-ray analysis carried out by Astbury (1937) and Astbury and Bell (1940).

In the main three methods have contributed most in further attempts to unravel the amino acid sequence of collagen.

The first of these methods, controlled partial acid hydrolysis, was first used by Grassmann and Riederle (1936) and this was followed by a series of papers in the early 1950's all using this technique, the major contribution being due to Kronor, Tabroff and McGarr (1953, 1955), Schroeder, Kay, Lewis and Munger (1955), Schroeder, Kay, LeGotto, Monson and Green (1954) and Heyns, Anders and Decker (1950). The techniques involved adaptation of the method of Sanger previously used for the determination of insulin structure (Sanger and Tuppy, 1951; Sanger and Thompson, 1953). Sequences of Glycine - Proline - Hydroxyproline - Glycine and of Glycine - Proline - alanine were

found to occur frequently in the hydrolysates, results which supported the periodicity hypothesis of Bergmann and Niemann (1936, 1937).

The second type of sequence determination involved the splitting of the molecule, after conversion to gelatin, using proteolytic enzymes of the noncollagenase type. Studies by Courts in 1955 indicated that Trypsin was a useful enzyme in this respect, the high lysine and arginine content of gelatin leading to the production of peptides of a useful size. He further found that over one third of the amino terminal groups of these peptides were glycine residues. Studies by Grassmann, Hannig, Endres and Riedel (1956, 1957b), using crystalline trypsin, demonstrated a splitting of the molecule into peptides, one third of the residues of which could be demonstrated as glycine. Further studies by Grassmann, Hannig and Schleyer (1960) led to the separation of 114 peptides, of which 55 were homogeneous. Furthermore, only lysine or arginine appeared as carboxyl-terminal amino acids and in nearly all the peptides glycine was the amino-terminal amino acid. Glycine appeared to occur as every third residue throughout the molecule. The interesting S_β peptide found in the prior determinations (Grassmann et al., 1956, 1957b and Grassmann, 1960) which contained two aspartic acid residues as the carboxyl-terminal portion was not found in the latter studies. This was possibly due to the presence of chymotrypsin contamination of the trypsin

in the first study which was removed by electrophoretic purification for the second study. In all these studies it became apparent that the overall picture given by sequence determination was one of apolar regions of high proline and hydroxyproline concentration alternating with polar regions containing few imino acids; in the latter there appeared to be subregions containing mainly acidic or basic amino acids. It was further suggested that the apolar regions corresponded with the "crystalline" interbands shown in Electron microscope and X-ray work, and the polar regions with the "amorphous" bands. Kuhn (1960) has proved that the polar regions are the binding sites for electron dense stain giving dark bands in the electron microscope. These results would partially refute the hypothesis of Bergmann and Niemann (1936, 1937). Finally, a number of peptides have been found to have a three chain configuration (containing two or three amino-terminal amino acids) which might represent positions of cross linking in the native molecule.

The third type of sequence determination involved the use of Collagenases, in particular the collagenase from Clostridium histolyticum, to break the molecule into peptides. Because of the defined specificity of clostridial collagenase, use of the enzyme in sequence studies has produced particularly useful results. Grassmann, Nordwig and Hörmann (1961b), Grassmann, Hannig and Nordwig (1963), Schroeder, Ogle and Logan (1959), and Gallop and Seifter (1962) have found that the major peptides were of the form Glycine - Proline - Hydroxy-

proline, Glycine - Proline - Alanine and Glycine - Proline - Glycine with smaller proportions of Glycine-Proline-Glutamic acid, Glycine-Proline-Serine and Glycine-Proline-Threonine. Nordwig, Hörmann, Kuhn and Grassmann (1961) confirmed, by electron microscope and chemical studies, that collagenase attacks the crystalline apolar regions of the molecule.

Thus it has become apparent that the crystalline apolar regions consist of sequences of the type Glycine-Proline-X, or to a lesser extent Glycine-Hydroxyproline-X, X being most frequently alanine, glycine or hydroxyproline; results which confirm Borgmann and Niemann's hypothesis.

The amorphous or polar portions of collagen are left relatively intact as peptides of an average chain length of 15 residues. The amino-terminal amino acids are almost all glycine due to collagenase specificity and glycine also occurs at every third residue. These peptides also contain most of the tyrosine and hexoses of collagen (Seifter, Gallop and Franzblau, 1961; Franzblau, Seifter and Gallop 1964; and Rojkind, Blumenfeld and Gallop 1964), and most of the aldehydic components, ester like bonds and γ-glutamyl linkages (Seifter and Gallop, 1966). The tolo peptides isolated by Rubin, Pfahl, Spoelmann, Davison and Schmitt (1963) would also be included in this type of fraction.

The Concept of the Tropocollagen Molecule.

Nageotte (1927) demonstrated that certain collagens after being dissolved in weakly acid media (0.00N acetic acid) could reform fibres on precipitation with sodium chloride. Fauré-Fremiet (1933) showed that similar results could be obtained with the collagen from the fish swim bladder. Nageotte and Guyon (1934) recognised the importance of the age of the animal from which the collagen was derived in further work and suggested that the extracted collagen was a precursor of collagen fibres in vivo, (the term Précollagène was introduced at this time).

The similarity of the wide angle X-ray diffraction patterns of native and precipitated collagen was demonstrated by Wyckoff and Corey (1936). These studies were extended further by Orekhovitch, Toustanovskii, Orekhovitch and Plotnikova (1948) to collagens of a variety of animal species and of animals of different ages within the same species, using acid citrate buffers. The results confirmed Nageotte's work and supported the concept that a more soluble precollagen was the precursor of the older more insoluble collagen, which dominated tissue structures.

These results, combined with those of Schmitt, Hall and Jakus (1942), using electron optical studies, led to the concept that a basic building unit exists for collagen; a view which is now generally accepted.

Exhaustive studies by Boedtker and Doty (1955, 1956) indicate a size and shape (Molecular weight 340,000; 3,000Å x

15Å) for the collagen molecule. These results confirmed the tropocollagen concept of Highberger, Gross and Schmitt (1951) and were consistent with the suggestions made by Gross, Highberger and Schmitt (1954), that the length of the collagen molecule is four times the major repeat distance 640-700Å observed in the native fibre by electron microscope and small angle X-ray studies.

THE AGGREGATION FORMS OF THE TROPOCOLLAGEN MOLECULE.

The work of Schmitt, et. al., (1942) and Wolpers (1943) showed that the collagen fibril was characteristically about 1,100Å wide and was further characterised by possessing a 640Å periodicity as seen with narrow angle X-ray diffraction or in the electron microscope after staining with phosphotungstic acid. The 640Å banding appeared to be constant for collagen fibrils from any source. Further studies showed that, in fact, the diameter of the fibril varied from 100Å - 1,000Å depending on the type of tissue source.

Intra period bands, that is banding within the 640Å period, were first described by Wolpers (1944). This author found two or three bands and as further work was carried out, Schmitt, Hall and Jakus (1945) described five and Hofmann, Nemtschek and Grassmann (1952) described ten. The bands appeared to have a fixed intra period position and density of phosphotungstic acid staining. Wolpers (1950) expressed the view that a normal mature collagen fibril had only two intra period bands and the presence of more bands was indication of pathological change. However this view is no longer held and the typical collagen fibril electron micrograph found with positive phosphotungstic acid staining (pH 4.2) is shown in Plate I.

It has been suggested that the collagen fibril has a tubular structure (Wyckoff 1952; Kennedy 1955; Haisa 1962),

the central core being packed with mucopolysaccharide. Smith, J.W. and Serafini-Fracassini (1967) have proposed a tubular fibril of 50Å diameter as a basic building unit of the larger fibrils. Staining with bismuth indicated that there were no sulphated mucopolysaccharides present in these fibrils.

Schmitt, Gross and Highberger in 1955 discussed the three forms of collagen arising from a study of soluble fractions in both acid and alkali and the factors concerned with precipitation of fibrils, by dialysis, salt concentration and mucopolysaccharides. Three forms of precipitated collagen were noted.

(1) The standard fibrous collagen, which has the characteristic 640Å spacing when 'stained' with phosphtungstic acid and viewed in the electron microscope.

(2) The fibrous long spacing type (F.L.S.) which was produced by precipitation with glycoprotein (Highberger et al., 1951). Other agents were found to produce this type, for instance, chondroitin sulphate, thrombin and heparin (Randall, Fraser, Fitton-Jackson, Martin and North 1952).

(3) The segment long spacing type (S.L.S.) produced by precipitation with adenosine triphosphate (ATP). (Schmitt, Gross and Highberger, 1953). The three types are shown in Plate I.

These studies indicated the complexity of collagen wit regard to ordered "crystalline" structures in vitro. Ramsey

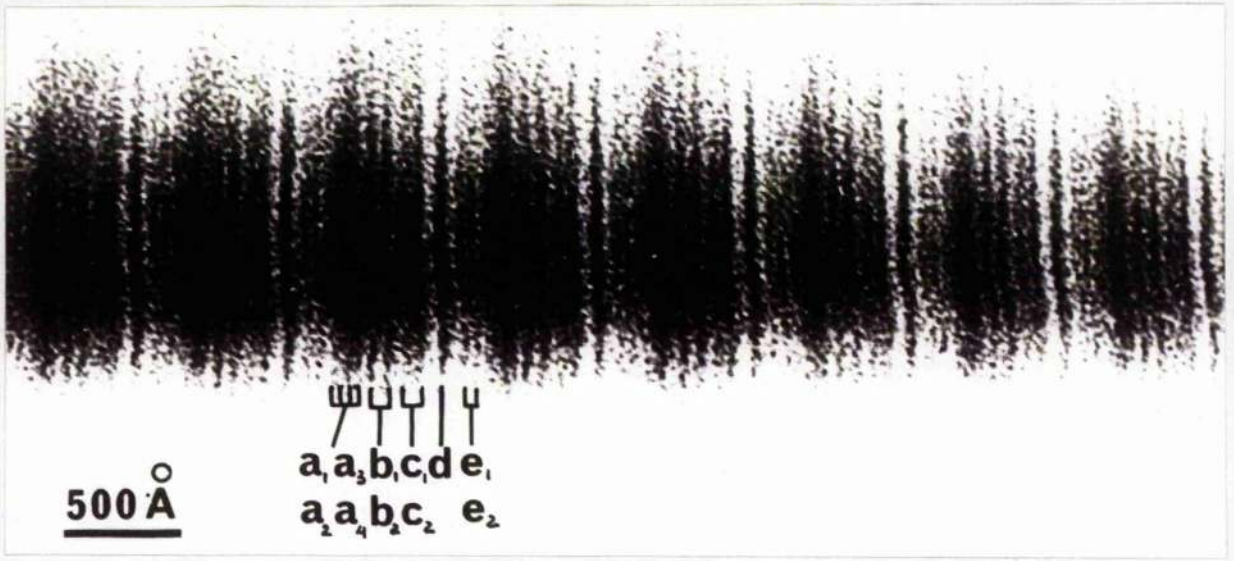
PLATE I

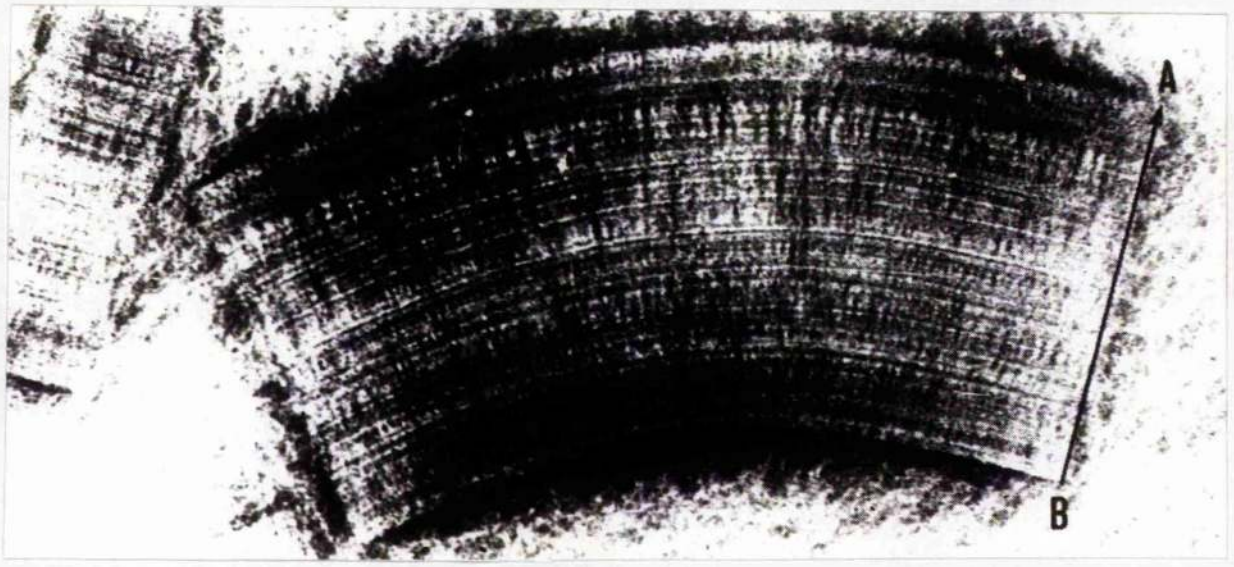
Aggregation Forms of Collagen seen under the Electron Microscope

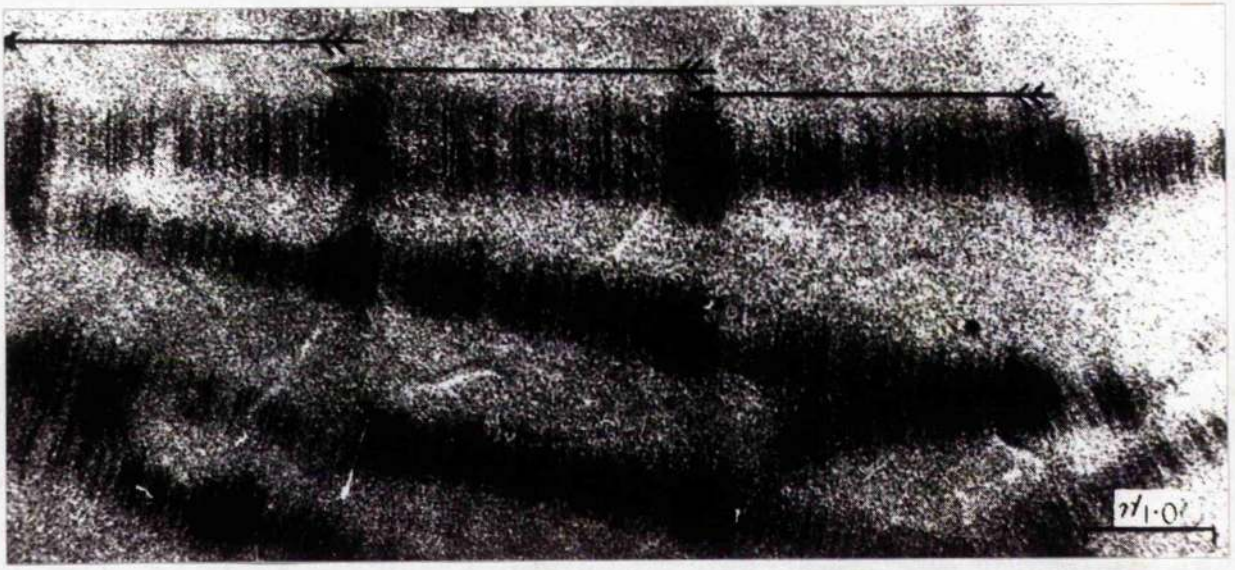
Top: The native type (positive PTA staining, pH 4·2).

Centre: Segment long spacing (S.L.S.) type (PTA staining, pH 7·0).

Bottom: Fibrous S.L.S. type (positive PTA staining).


a1 a3 b1 c1 d e1
a2 a4 b2 c2 e2
500 Å


A
B



(1965) has suggested the presence of the F.L.S. type 'in vivo' in samples of tissue from brain tumours.

The electron microscope appearance of these forms of collagen shows an alteration of two kinds of region characterised by different affinities for acidic and basic electron optical 'stains'. These appear as bands and interbands of different size. These observations would support the proposal of Bear (1952), based on investigations using narrow angle X-ray diffraction techniques, that the molecule contained alternating regions of polar and non polar character. Evidence supporting this was the enzymic degradation of the molecule into a group of peptides which were largely polar and a second group which were largely non polar (see work of Grassmann and his colleagues; Nordwig et al., 1961).

Tropocollagen molecules in the native banded structure have been suggested to occur in an arrangement in which one molecule is displaced by a quarter of its length with respect to a second (the Quarter Staggered arrangement; Hodge and Schmitt 1960). The individual molecules are all arranged in the same direction so that the overall arrangement is polar, i.e there is a 'head' and 'tail' end.

Schmitt and Hodge (1960) and Hodge, Highberger, Deffner and Schmitt (1960) have indicated the existence of 'telopeptides' (non helical terminal peptides) in the tropocollagen molecule. Hodge et al., (1960) showed that the majority of the tyrosine

in the molecule was present in these appendages and that the action of trypsin and chymotrypsin led to a molecule which would not polymerise to the native type fibre. It was suggested that these appendages were involved in the end to end polymerisation and furthermore Bensusan and Scamu (1960) and Steven (1965a) have suggested the involvement of the tyrosine residues in cross linking reactions during fibril formation. Kuhn, Kuhn and Hannig (1961) found that polymerisation would only occur very slowly after treatment with trypsin or chymotrypsin and they attributed this to proteolytic decomposition of accompanying impurities of connective tissue collagen.

However, Rubin et al., (1963) and Schmitt (1964) stressed the significance of alterations in the behaviour of tropocollagen due to the protease cleavage of the telopeptide; (Pepsin was used in these studies). They showed, in agreement with Nishihara and Miyata (1962), that the telopeptide appeared to be responsible for the polymerisation and fibrogenesis of collagen and that it appeared to be the region of the molecule containing the cross links visualised by Piez and his co-workers to be involved in the formation of the β dimer and γ trimer from α monomers (see later). Further studies (Drake, Davison, Bump and Schmitt 1966; Rubin, Drake, Davison, Pfahl, Speakman and Schmitt 1965) on the telopeptide concept have shown that removal of the telopeptide caused alterations in the antigenic properties and aggregation properties of the tropocollagen molecule but

that no changes were discernible by electron microscope examination.

Hörmann and Hafter (Hörmann and Hafter 1963; Hafter and Hörmann 1963) have continued the work of the German group and confirmed that after treatment with pepsin, the reduction in fibril formation can be attributed to the destruction of non collagen impurities which were essential for the formation of fibrils.

Kuhn and Eggl (1966) and Kuhn, Fietzek and Kuhn (1966) have shown that after treatment with pronase there is an apparent shortening of the molecule (as shown by electron microscope observations on the S.L.S. aggregation type) amounting to about 150Å (5%) from the A end only. After such treatment the molecule was susceptible to pepsin or trypsin digestion, and it was suggested that pronase removed a portion of the molecule which blocked attack by most non collagenase proteases. Furthermore, Kuhn, et al., (1966) distinguished between two types of inter molecular bond, one responsible for side to side aggregation and stabilisation of the quarter stagger arrangement and a second responsible for end to end polymerisation, the side to side type of bond was more susceptible to pepsin attack than the end to end bond.

Recently Petruska and Hodge (1964) using a negative staining technique and high resolution electron microscopy have shown that the tropocollagen units in the native fibrous form were not linked end to end but that hole zones of about 0.6 of

the molecule overlap distance interposed.

Some work by Grant, Horne and Cox (1965) using negative staining techniques in electron microscopy has led these authors to suggest also that there was no requirement for end to end linkage of the tropocollagen molecule through 'tails'. They conceived the tropocollagen molecule as consisting of a rather flexible filament divided into nine zones, five of which were bonding zones and four were non bonding.

On the basis of the obvious three dimensional structure of a collagen fibril the validity of the two dimensional quarter stagger arrangement has been questioned by Ross and Benditt (1961), McGavin (1964) and Smith, J.W. (1965). Smith J.W. and Serafini-Fracassini (1967) have further suggested a model for a basic building unit of the collagen fibril which would fit in with the quarter stagger arrangement. They visualised a tubular structure of 50Å diameter made up of a series of eight tropocollagen molecules surrounding a central hole. Further measurements indicated that fibrils could, in fact, be seen having a 30Å diameter, possibly made up of a series of four molecules which would eliminate the need for a large central hole in the structure. In these structures each molecule is only in contact with two other molecules, which satisfies the quarter stagger hypothesis, (see Smith, J.W. 1965).

The Secondary, Tertiary and Quaternary Structure of Collagen.

The actual molecular structure of collagen which is accepted at the present time is the triple helix structure. This was suggested in a series of papers by Ramachandran and his colleagues in the mid-1950's (see Ramachandran and Kartha, 1954, 1955; Ramachandran 1956) in which they proposed that the 2.86Å meridional reflection described a repeat unit in a system of coiled coils formed by three left handed mirror helices winding in a right handed helix about a common axis. Further extensions of this concept were presented by Cowan and McGavin (1955) Cowan, McGavin and North (1955), Bear (1956), Crick and Rich (1955) and Rich and Crick (1955, 1958, 1961). Cowan *et al.* (1955) used poly-L-proline as a synthetic model for X-ray work while Crick and Rich (1955) used polyglycine II.

It was generally accepted that only two models could be constructed which were compatible with all the physical data and these were termed structures I and II (Rich and Crick 1958). Both models contained three left handed helical chains. Each chain was considered to have a three fold screw axis i.e. from X-ray data the projected length of one residue was 3Å and the translation for one complete turn was 9Å (3 residue lengths), each residue twisting through -120°. In the complete structure the three chains were aligned along parallel axes in such a manner that every third peptide group in a chain occurred in an environment identical with that of every other third peptide group.

Each model was capable of forming the same numer of hydrogen bonds, the difference between the two models being the phasing of the chains with respect to each other. To illustrate the difference in phasing, a convention was adopted to call position 1. that peptide bond in a single chain which is directed toward the centre of the three chain complex. Positions 2. and 3. were the next two peptide bonds in that chain. These positions obviously repeated along the length of a chain. In structure I the three chains were phased so that hydrogen bonds might occur between the -NH of position 1 peptide bonds of one chain and the -C=O of position 1 peptide bonds of the neighbouring chain. In structure II, hydrogen bonds could occur between the -NH of position 1 peptide bonds of one chain and the -C=O of position 2 peptide bonds of the neighbouring chain. A diagrammat: representation of structures I and II is shown in Plate II.

These models discussed so far have not taken into account the side chains of residues composing the polypeptide chains. The accommodation was expressed in slight deformation of the chains so that they were no longer parallel but instead formed a major right handed twist to the whole structure; (see Plate II). The dimensions for such a model were slightly different from those mentioned above. A rotation of -108° described the translation for a residue on one chain to a similar residue in the neighbouring chain, the distance being 2.86Å. A rotation of -324° in a distance of 8.6Å culminated in a residue in the original chain.

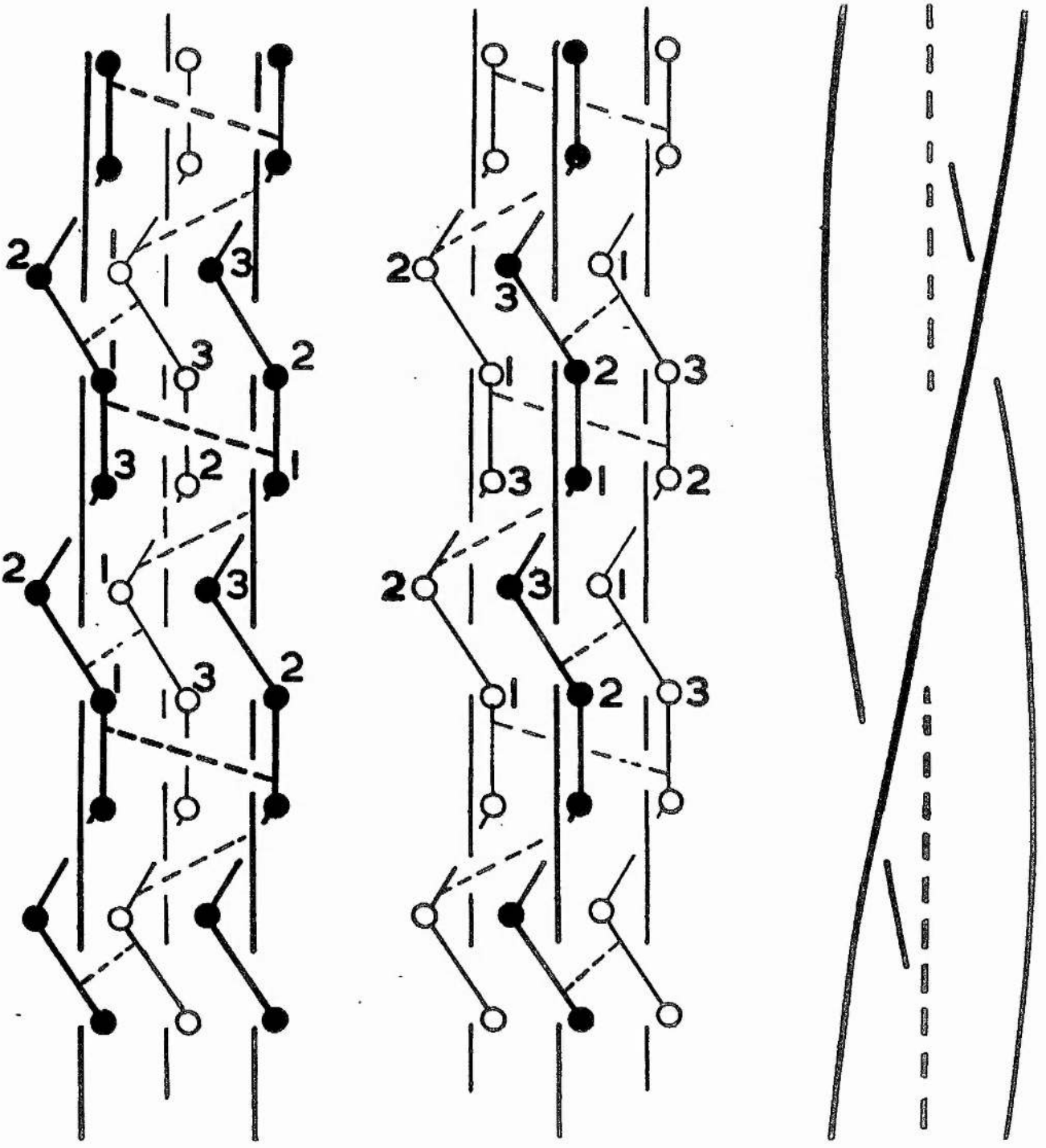
PLATE II

A Diagrammatic Representation of the Structure of the Collagen Molecule

Left hand figure: The Collagen I type of structure. The open and full circles represent α-carbon atoms of the amino acid residues. The dotted lines represent hydrogen bonds.

Centre figure: The Collagen II type of structure.

Right hand figure: An indication of the general way in which the two structures may be deformed to form the tropocollagen model. The solid lines represent the axes of the three polypeptide chains which have a gradual right hand twist. The broken line represents the common axis around which the three chains twist.


2
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3
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2

Table 3 shows the major differences between structures I and II. In particular differences in position 3 of the tripeptide sequence are important. In structure I only glycine can be involved in an undeformed model, whereas in structure II any amino acid, including the imino acids, may be involved. On this basis, bearing in mind the tripeptide sequences found in enzymic and acid digests of collagen, structure II has been favoured as the most likely structure until fairly recently.

Ramachandran, Sasisekharan, and Thatchachari (1962) and Ramachandran (1962, 1963) have proposed a third type of structure which seemed to fit in to the accepted data better than either structures I or II. The basic difference of this "Madras" model is that it had two hydrogen bonds per triplet instead of one hydrogen bond as in the Rich and Crick structures. It should be noted that where proline and hydroxyproline occur together with glycine in a triplet then the Madras model cannot operate, as imino acids cannot form hydrogen bonds of the type visualised.

The actual collagen structure may conform to one of the three models proposed or perhaps to a distribution of all three depending on the order of the side chains, determined, in turn, by the primary amino acid sequences of the single chains.

TABLE 3

Possible positions of side chains in Collagens I and II

	Collagen I		Collagen II
Position	Undeformed	Deformed	
1.	Gly. only	Other residues may be possible; pro or hypro impossible	Gly. only
2.	Any residue including pro. or hypro.	Any residue including pro. or hypro.	Any residue cluding pro. or hypro
3.	Gly. only	Any residue including pro. or hypro. except valine	Any residue cluding pro. or hypro.
Bonding of the -OH of hypro in position 3.		Can make a hydrogen bond to the neighbouring chain <u>within</u> the group of three chains	Projects radially awa from the structure an <u>cannot</u> make hydrogen bon <u>within</u> the group of thr chains
Position of the inter-chain hydrogen bonds.	From the =NH of residue 1. to the = CO of residue 1. on a neighbouring chain		From the =NH of residue 1 to the =CO residue 2. a neighbouring chain.

Rich and Crick (1958).

The Internal Structure of the Tropocollagen Molecule

The denaturation of tropocollagen under conditions sufficiently mild to assure no breakage of peptide bonds, leads to definite and easily discernible physical changes. It was found that the molecular weight decreased to a definite "end point" and the final product was termed a "parent gelatin", (Gallop 1955, Cohen 1955) the end point molecular weight being about 70,000.

Orekhovitch and Shpikiter (1955, 1958) found that ultra-centrifugation of denatured tropocollagen from rat skin led to a separation of the "gelatin" into two components, one heavy (labelled β) and one light (labelled α). These authors further formed the view that the components α and β existed naturally in a 1:1 ratio.

Boedtker and Doty (1956) in investigations of the collagen from carp swim bladder (ichthyocol) showed that the molecule split into three subunits on denaturation. Furthermore X-ray studies on collagen had indicated that the crystalline regions probably consisted of three chains, each wound into a helix resembling poly-L-proline. It was suggested that these subunits were the individual chains of collagen.

Investigation of the size and shape of these components (Chun and Doty 1958; Doty and Nishihara 1958) led to finding that the β component was about twice the molecular weight of the α component. Occasionally a third, heavier, component was noted.

In 1959 and 1960 Kessler, Rosen and Levenson introduced column chromatography at 40°C on Carboxy-methyl-cellulose (CM-Cellulose) and then Piez, Weiss and Lewis (1960) carried out the fractionation of the α and β components using this system with an ionic strength gradient. Two types of α, α_1 and α_2, and two types of β, β_1 and β_2, were observed. Orekhovitch, Shpikiter, Masurov and Kounina (1960) proposed that the β component could be two α components condensed together by an ester bridge. Piez, Lewis, Martin and Gross (1961) expanded this further with a series of experiments on salt extracted and acid extracted collagen. From the ratios of the two types of subunit present in both cases, and their molecular weights, they suggested that the β components were chain pairs joined by one or more cross links, and, furthermore, that there were two α_1 subunits and one α_2 subunit per molecule of collagen, these forming the β_2 subunit by the joining of two α_1 subunits, and the β_1 subunit by the joining of one α_1 and one α_2 subunit in the more mature forms of collagen i.e. acid soluble collagen. Salt soluble collagen contained little or none of the β component (Piez et al., 1961; Piez, Eigner and Lewis 1963). Grassmann, Hannig and Engel (1961a) described the presence of a third component now known as the γ component and this, or a similar component, was also described by Wood (1962).

Investigations on the reversion of α, β and γ components to collagen have indicated that the γ component with all three chains linked together underwent the most ready and

complete return to the collagen structure (Veis, Anesey and Cohen 1961). Presumably the chain adjacent to the cross links was held in the correct configuration and acted as a nucleus for reversion.

The presence of such subunits in the collagen molecule demonstrated the presence of intramolecular cross-linking. The isolation of larger components, however, (Veis, Anesey and Cohen 1960; Veis et al., 1961) demonstrated that intermolecular cross-linking also occurred. This latter type of cross linking was discussed by Bornstein, Martin and Piez (1964) and Bornstein and Piez (1964) after their isolation of a β component which was apparently a dimer of two α_2 chains, a situation which could arise only by intermolecular cross linking.

The components of the collagen molecule are now labelled as follows:-

α_1; α_2; β_{11} (β_2); β_{12} (β_1); γ_{11}

The intermolecular species is, of course, β_{22}.

Piez (1964 and 1965) described a third α component in the fractionation of cod skin collagen, which was similar to the α_1 component. Heidrich and Wynston (1965) isolated a similar α_3 component from calf and rat skin collagen. However, Piez (1967) stated that from his experiments on the splitting of α chains by cyanogen bromide (CNBr) there appeared to be only two types of α subunit in rat skin collagen, but the existence of α_3 in calf skin collagen has not yet been disproved.

Finally it has become clear that the formation of at least two discrete components on denaturation of acid soluble collagen is a property of collagen from all species. Examples of sources used in such experiments include: rabbit skin, rat skin, calf skin, human skin, rat tail tendon, carp ichthyocol, cod skin, dog fish shark skin and spiny dog fish skin (Harding 1965).

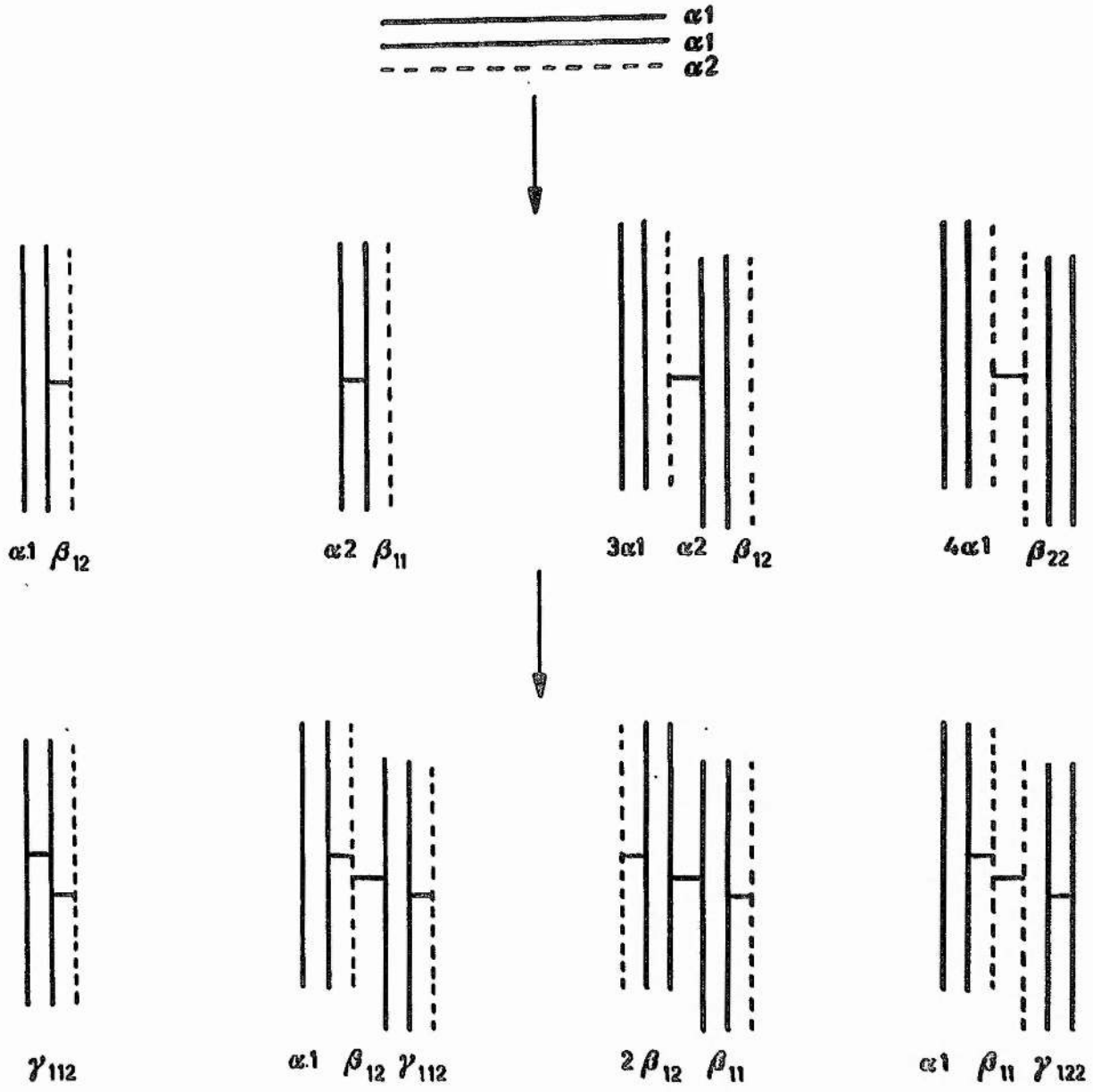
PLATE III

A Diagrammatic Representation of the Early Stages of Collagen Cross-linking.

Top: The triple-chain molecule is initially not cross-linked. One of the three chains (α_2) is different from the other two (α_1).

Centre: One cross-link produces β-components (β_{11}, β_{12} and β_{22}) which can be isolated from the denatured collagen. Additional products can arise from either inter- or intra-molecular cross-linking.

Bottom: Additional cross-linking produces larger aggregates including γ-components.
Cross-linking may continue to give rise to a continuous three dimensional structure.


α1
α1
α2
α1 β12
α2 β11
3α1 α2 β12
4α1 β22
γ112
α1 β12 γ112
2β12 β11
α1 β11 γ122

Subunits of α chains

Gustavson (1956) considered the possibility of the presence of ester groups in collagen due to the appearance of carboxyl groups in excess of amino groups in alkali treatment of gelatin. Some support for this suggestion was derived from investigations of Grassmann, Endres and Steber (1954) and Konno and Altman (1958). These authors noted the formation of a variety of amino alcohols on reduction of collagen and gelatin with lithium borohydride, a reaction which would occur with ester groups, although the variety of amino alcohols which were formed did not suggest the presence of any one particular ester.

Experiments on more highly purified collagen by Gallop, Seifter and Meilman (1959) using hydroxylamine or hydrazine as a nucleophilic agent for the cleavage of ester bonds provided evidence for the presence of ester like groups (although the possibility existed that they could be special imide bonds, although this was considered doubtful). The action of one molar hydroxylamine at a pH of 9-10 was to cleave denatured tropocollagen leading to a reduction in molecular weight from approximately 100,000 → 20,000, with the formation of 5-6 protein bound hydroxamic acid residues per 100,000 unit. When hydrazine is used a similar reaction ensues in which six protein bound acid hydrazine groups are formed. (These techniques have also been used in other laboratories; see Bello 1960; Hörmann 1960b.)

Since these reactions involved the approximately five fold decrease in molecular weight, it was presumed that the ester like

groups being broken cannot involve groups of small molecular weight in linkage with either carboxyl, hydroxyl or amino groups of side chains of bifunctional amino acid residues of the protein backbone (Seifter and Gallop 1966). Such groupings were visualised, for example, as simple hexoses in single ester linkag with a distal carboxyl group of a glutamyl or aspartyl residue.

These studies by Gallop et al., (1959) were carried out before elucidation of the tertiary structure of collagen and recognition made of the α and β components as separable entities. Thus treatment of denatured collagen would lead to a mixture of subunits particularly if the subunits of α_1 and α_2 chains were different.

Seifter and Gallop (1963) and Gallop (1964), on the basi of results suggesting ester like bonds to occur in pairs (Blumenfeld and Gallop 1962), considered that each α chain consists of four subunits held together by three pairs of ester like bonds

Further studies on the separation of the subunits of the chains on Sephadex and polyacrylamide (Blumenfeld, Rojkind and Gallop 1965) led to a finding of components of three different sizes having molecular weights of 17,000, 34,000 and 50,000, the first two components being present in greatest amounts. This le Gallop (1966) to put forward his 3:2:1 A B C subunit hypothesis i which he suggested that there were six subunits to each α chain. He based his hypothesis on the finding by Pies (1964) that there were three types of α chain in cod fish skin collagen and the suggestion that this might be universally the case in all collage

Gallop visualised the subunits as being of three types, A, B, C and the α chains being made up in the following manner.

$$\alpha_1: 3A + 2B + C$$

$$\alpha_2: 3C + 2A + B$$

$$\alpha_3: 3B + 2C + A$$

These data fit in with amino acid analysis data and also the common occurrence of the number 6 or multiples thereof in various aspects of collagen chemical make up.

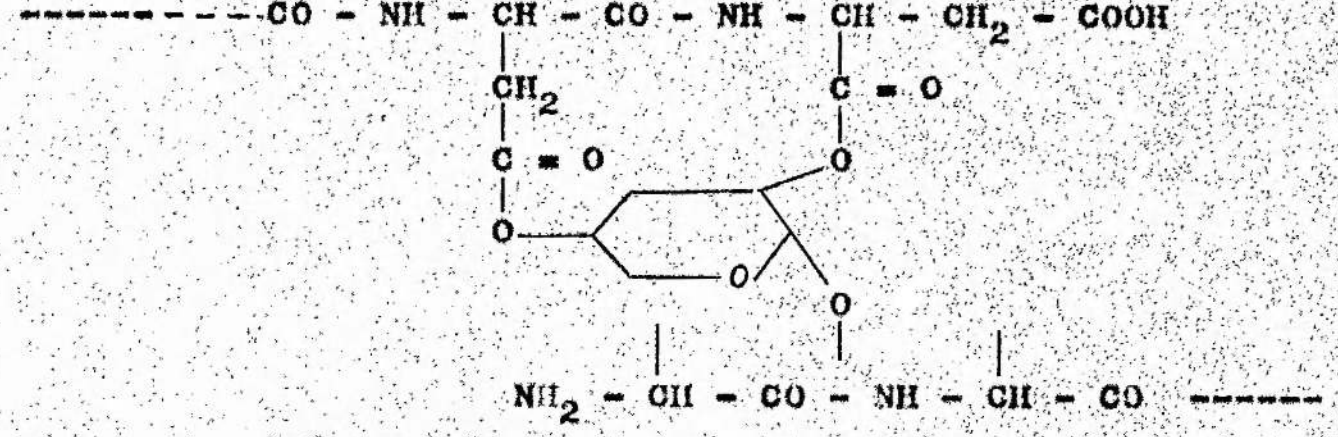
This suggestion did not detract from the previous statement of the existence of three paired ester like bonds. It was visualised that there were two undefined covalent types of link present in the molecule (Seifter 1966) which would possibly lead to the occurrence of the 34,000 molecular weight component as well as the 17,000 molecular weight component.

Petruska and Hodge (1964) using electron microscope studies on S.L.S. collagen, and Bailey and Hodge (1965) and Hodge, Petruska and Bailey (1965) treating separate α chains with hydroxylamine followed by chromatographic separation showed evidence for a 5-5-7 subunit model, α_1 chains containing five subunits of molecular weight 20,000, and the α_2 chain seven subunits of molecular weight 14,000. Petruska (1966) has suggested a 5-5-6 model as more fitting with recent data.

Further evidence for subunits of α chains was provided by the detection of acetyl groups in collagen by Hörmann and

FIG. I.

The configuration of the intra-chain subunit cross-link as visualised by Gallop (1964). Carbon 1 of the unidentified hexose is assumed to be in glycosidic linkage with a side chain hydroxyl function on the adjacent subunit chain.



Joseph (1965). Hörmann, Joseph and von Wilm (1965) demonstrated six moles of acetyl group per 1,000 moles of amino acid which were linked to α-amino groups. These results indicated the presence of six subunits per α chain with their amino terminal groups acetylated. It is interesting to note that other proteins have been shown to have amino terminal groups substituted in a similar way; for example, rabbit muscle enolase (Winstead and Wold 1964), myosin (Offer 1964), muscle α glycero-phosphate dehydrogenase (van Eys, Judd, Ford and Womack 1964).

The fact that the linkage joining these subunits together was ruptured by treatment with hydroxylamine and hydrazine was proof that one half of the linkage was provided by an acyl moiety. If the linkage was of the ester type then the other half would be an alcohol grouping (if of the special imide type the other half would be an amide grouping).

From the work of Blumenfeld and Gallop (1962) it has become apparent that the acyl functions were provided by two aspartic acid residues at the carboxyl end of the subunit. The linkage is visualised by Gallop (1964) to be of the type shown opposite, with the α carboxyl of one aspartic acid and the β carboxyl of the second aspartic acid possibly linked to an unidentified carbohydrate (possibly glucose or galactose). Other hydroxyl functions which might be involved include those of 3-hydroxyproline (recently shown to be present in collagen by Ogle, Arlinghaus and Logan (1962), 4-hydroxyproline, hydroxyly

sine, tyrosine, threonine and serine. The hydroxyl group of tyrosine is probably not involved as this function was shown to be free in collagen (Franzblau, et al., 1964). Recent work on the function of hydroxylysine (Butler and Cunningham 1965, 1966) indicated that a small proportion was substituted at the hydroxyl function by a disaccharide consisting of glucose and galactose. However the majority of the hydroxylysine (95%) has been shown to be destroyed by periodate oxidation indicating no substitution of the hydroxyl group (Blumenfeld, Paz, Gallop and Seifter 1963; Schlueter and Veis 1964; Hörmann and Fries 1958). Thus the involvement of this and other hydroxyl functions appears to be still a matter for speculation.

Piez (1967) suggested, from his results on cyanogen bromide cleavage of α chains, and Kang, Nagai, Piez and Gross (1966) from studies on the cleavage of tropocollagen by a collagenolytic enzyme from the tadpole, that each chain was unique through its entire length so that there could be no type of repeating intra chain subunit, although the existence of non identical types of intra chain subunits could not be ruled ou

Kuhn, Tkocz, Zimmerman and Deier (1965) have recently shown that a typical long-spacing segment aggregation of collagen can be formed from renatured α_1 chains. These authors suggest, on the basis of this finding, that the subunit model due to Hodge and his colleagues (Petruska and Hodge 1964; Bailey and Hodge 1965; Hodge, Petruska and Bailey 1965), the 5-5-7 or 5-5-6 model or for that matter the model proposed by Gallop (1966), the 3-2-1

ABC model, could not be tenable for obvious reasons. However, as Seifter and Gallop (1966) pointed out, it was possible that α_1 chains in the absence of α_2 chains could form a stable aggregate in a similar way to that formed by abnormal haemoglobins, (e.g. Haemoglobin H), which result from association of varying numbers of α and β chains (Haemoglobin H consists of four β chains).

The Stability of Collagen

The stability of a fibrous macromolecule will obviously depend on its ability to retain the native shape, and this will depend on a number of factors including the steric structure (related to the amino acid sequence) and content of cross links of the molecule. Harding (1965) has stressed the necessity of cross linking as a prime factor in the stabilisation of the tertiary and quaternary structure of proteins, configurations which are essential for biological activity. Both Chemical and Physical methods have been used in the elucidation of the types of structures influencing the stability of collagen. The most important physical method has probably been that of the determination of the shrinkage temperature of collagen fibres. This technique has been adapted from the tanning industry where it was, and is, used in the determination of the extent of tanning (cross linking) of leather. (The term will be used without introduction in this section; for a summary of the theory behind the technique the reader is referred to page 99). The technique has been used in the estimation of the correlation of the stability of collagen and its imino acid content, a topic which will now be discussed.

The Stability of Collagen as measured by its shrinkage Temperature (Ts) and its relationship to the imino acid content.

From investigations carried out by Gustavson on the physico-chemical behaviour of collagen from various sources, it became apparent that the collagen from the skin of teleosts had

a low degree of hydrothermal stability when compared with that of mammalian skin (Gustavson 1942a & b; see also Gustavson 1962) the shrinkage temperature (Ts) was about 40°C for cod skin collagen, 58°C for eel skin collagen and 65°C for bovine skin collagen, and furthermore, compared with bovine skin collagen the fish collagen had a very low resistance to hydrogen bond breakers and swelling and solubilising agents. These findings were suggested to indicate a higher degree of intermolecular bonding for the bovine collagen. With the publication of the amino acid composition of bovine skin collagen (Bowes and Kenton 1948; Tristram 1949) and fish skin collagen (Neuman 1949) it became apparent that there were quite large differences in the imino acid (proline and hydroxyproline) content of these collagens. Hydroxyproline was present in teleostean skin in varying amounts from 7% - 11% whereas the hydroxyproline content of bovine skin was comparatively constant at 12.5% - 13%. Gustavson (1953, 1954) and Takahashi (Takahashi and Tanaka 1953; Takahashi and Yokoyama 1954) have independently indicated a direct correlation between the hydroxyproline content and Ts of fish skin collagen and bovine collagen.

These studies have led Gustavson (1955c) to propose the importance of the hydroxyproline residues in the stability of collagen. He considered that hydroxyproline will form interchain hydrogen bonds between its hydroxyl group and the carboxyl oxygen of an adjacent peptide group and that it was rupture of these

bonds which precipitated thermal shrinkage. This amino acid might also be involved in the formation of ester links between polypeptide chains. Evidence in favour of the hypothesis includes the observation that complete N-acetylation of bovine collagen did not affect the Ts, but combined N- and O-acetylation, blocking both amino and hydroxyl groups, lowered the Ts by about 20°C. (Other evidence was summarised by Gustavson 1956 and 1957).

A report has been published by Rigby and Spikes (1960) which further confirmed the relationship of Ts and Hydroxyproline content. They included results of Takahashi, and values for cattle hide and rat tail tendon as well as values for human dermal collagen which were quoted by Hall and Reed (1957). The overall picture is a variation of Ts from 20°C to 80°C with an increase of hydroxyproline from 4.5% to 15%.

In a review on the structure of collagen and gelatin, Harrington and von Hippel (1961) stated that Gustavson had dismissed the apparently equally good correlation between either proline or total imino acid content of collagens and their shrinkage temperatures on the grounds that proline cannot participate as a hydrogen donor in hydrogen bonding. Gustavson, in fact, apparently felt that the presence of proline would, if anything, reduce rather than increase the stability of collagen.

It became evident, however, that hydrogen bonding might not be the sole factor involved in stabilising collagen. Evidence was put forward that the pyrrolidine ring, which cannot

be incorporated into the α helical structure, might influence the configuration of polypeptide chains which contain proline and/or hydroxyproline and that they would be more likely to take up a poly-L-proline II type of structure, stabilised by restriction to rotation of the pyrrolidine ring about the peptide backbone (Szent-Gyorgyi and Cohen 1957; Harrington and Sela 1958; Harrington 1958). In studies on the gelatin - collagen fold transition, von Hippel and Harrington (1959) proposed that the poly-L-proline II type configuration develops along single gelatin chains as an intermediate step in the formation of the collagen type structure. This suggested, specifically, that restriction to rotation about the $-C_{\alpha}-C=O$ bond and the peptide bonds adjacent to pyrrolidine rings was involved in the stabilisation of the collagen configuration.

In comparisons of the dilute solution denaturation temperature (T_D) of various collagens, Burge and Hynes (1959) found a correlation between proline, hydroxyproline and/or total imino acid content and T_D, although the correlation with total imino acid content appeared to be the best of the three. Piez and Gross (1960) found statistically significant correlation of Ts and proline, hydroxyproline and total imino acid content in many collagens and suggested that the relation between Ts (and T_D) and total imino acid content is the significant factor; thus confirming Burge and Hyne's (1959) results. (See also Piez 1960)

Watson and Silvester (1959), studying the cuticle collagens of Lumbricus and Ascaris found that there was no relationship between Ts and the hydroxyproline content. However, Bauer and Rice (1963) demonstrated a correlation of total imino acid content and T_D in Lumbricus cuticle collagen. Josse and Harrington (1964) demonstrated that this relationship held both for Ascaris cuticle collagen and Lumbricus cuticle collagen, and several other soluble collagens.

It thus appears that the imino acid content contributes to the stability of collagen by virtue of its steric structure and also to a certain extent by virtue of the hydrogen bonds involving the hydroxyl group of hydroxyproline.

If I may refer back to the types of structure quoted earlier for the configuration of the residues and chains of the collagen molecule it will be remembered that structures I and II were suggested, along with a third type suggested by Ramachandran and his colleagues, the "Madras" structure.

Structure II was favoured due to sequence studies indicati an occurrence of hydroxyproline in position three of the triplet where it could occur without deformation.

Structure I, on the other hand, on the basis of the inward facing hydrogen bonds and the original concept of the relationship of shrinkage temperature and hydroxyproline content, was favoured by Doty and Nishihara (1958).

However, since the finding of the relationship between

total imino acid content and shrinkage temperature, Josse and Harrington (1964) and Harrington (1964) have demonstrated that data fit the Madras model most satisfactorily.

Covalent Cross-linking.

The evidence available for covalent cross links which might occur in mature collagen thus conferring stability has been reviewed recently by Harding (1965).

Gustavson (1946) suggested that electrostatic cross links were not the only cross links present in collagen due to the finding that treatment with 0.1M β-naphthalene sulphonic acid, blocking electrostatic links, led to a comparatively small fall in the shrinkage temperature.

More direct evidence for the existence of covalent cross links was supplied by Wiederhorn and Reardon (1952) from experimen on the stress strain characteristics of heat shrunk collagen. They calculated that a chain length corresponding to a molecular weight of 55,000 existed between each cross link.

Gustavson (1955a, b, c and 1956) proposed that there might be two types of covalent cross links existing in collagen:- (1) Inter chain ester links which were assumed to be formed between hydroxyl and carboxyl groups on adjacent chains. (2) Cross links involving the guanidyl groups of arginine assumed to be in part combined with carboxyl groups of adjacent chains.

Ester type links

Schneider (1949) first proposed the existence of ester linkages in collagen as a means by which sugar bridges were incorporated into the peptide chain. The more recent studies indicating the involvement of ester links in the intra chain subunits has already been discussed under the heading, Subunits

of the α Chains. Intermolecular and inter chain cross links are visualised to form by a process of trans-esterification (Gallop 1964; Verzar 1963b) see Plate IV.

Arginine cross links

A cross link involving a guanidyl - carboxyl bond has been strongly implicated by Gustavson (1955a, b). Joseph and Bose (1962) demonstrated that only 65% of the arginine of mature collagen was available to destruction by hypobromite. Kenchington and Ward (1954) using the indirect estimation from titration curve data found no masking of these groups in gelatin. It would seem that there is no direct evidence for the existence of this type of cross link at the present time; the results being due to a masking of the grouping in native collagen.

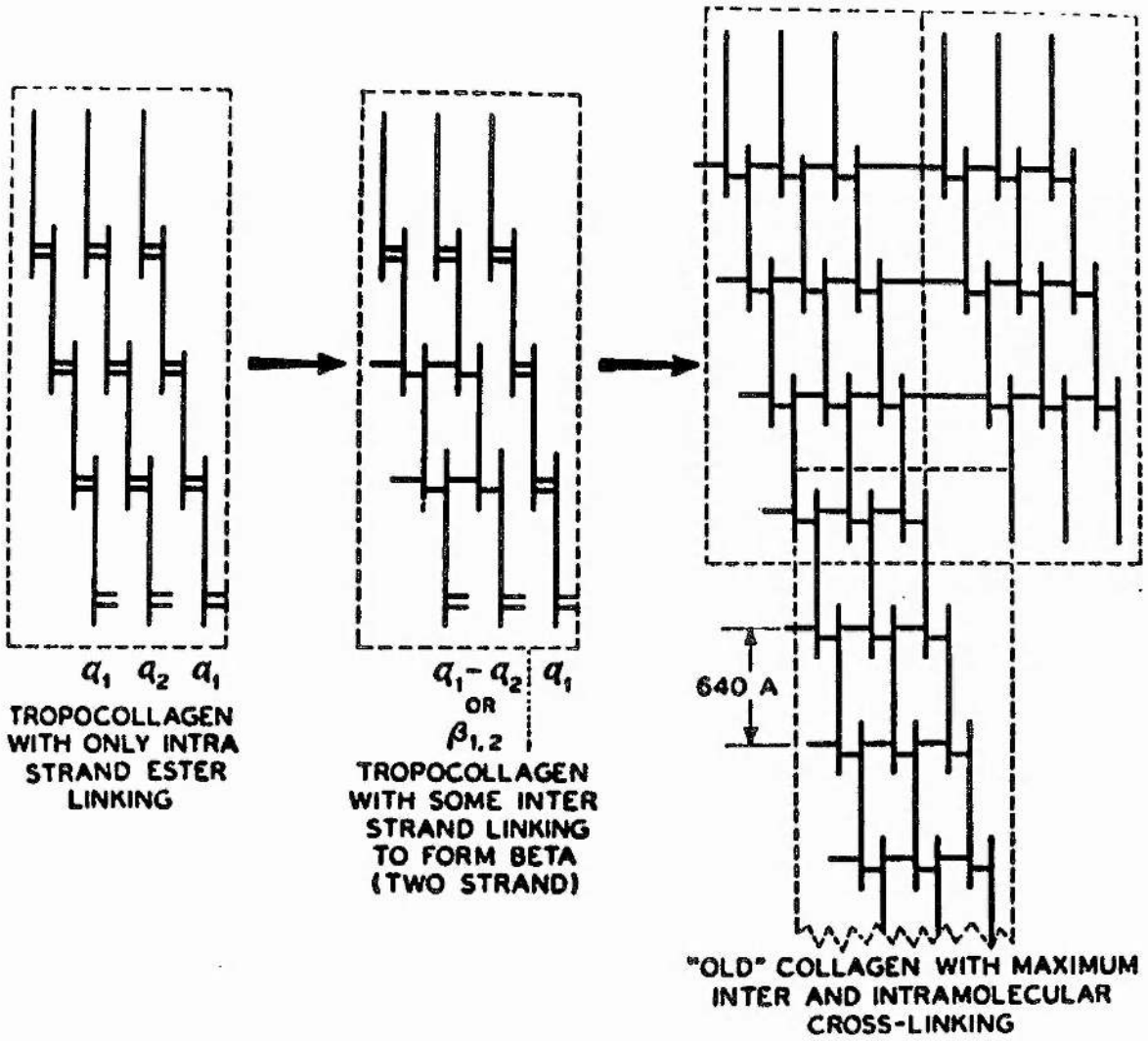
γ Glutamyl and β-Aspartyl linkages

Haurowitz, Zimmerman, Hardin, Lisic, Horowitz, Lietze and Bursa (1957) first suggested a γ-glutamyl link on the basis of the reaction of free α-carboxyl groups with thiocyanate to give thiohydantoin rings in the chain. However Deasy (1956) an Joseph and Bose (1959) found that thiohydantoins were formed by at least three other amino acids at temperatures much lower than those used by Haurowitz and his colleagues. The results of Franzblau (1962) and Franzblau, Gallop and Seifter (1963) indicated that γ-glutamyl links were present in the peptide chains of collagen and that β-aspartyl linkages were absent. Th oxidation of terminal γ-glutamyl residues to succinic acid afte

PLATE IV

Schematic Diagram to Represent the Maturation Process in Collagen.

Both Intra- and Inter-molecular Cross-linking are Shown Occurring by a Process of Trans-esterification with no Increase in the Total Ester Links.


α_1 α_2 α_1
TROPOCOLLAGEN WITH ONLY INTRA STRAND ESTER LINKING
α_1-α_2 α_1
OR
$\beta_{1,2}$
TROPOCOLLAGEN WITH SOME INTER STRAND LINKING TO FORM BETA (TWO STRAND)
640 A
"OLD" COLLAGEN WITH MAXIMUM INTER AND INTRAMOLECULAR CROSS-LINKING

enzyme treatment of gelatin and collagen led Joseph and Rose (1960 and 1962) to suggest that glutamic acid was present in collagen either as a γ-glutamyl cross-link or as a γ-glutamyl link in the peptide chain. However there appears to be no direct evidence for the existence of γ-glutamyl cross-links in collagen. In fact Harding (1965) stated categorically that the γ-glutamyl residues in collagen were not used in cross-linking.

ϵ-Amino linkages

The availability of ϵ-amino groups of lysine in gelatin and collagen to fluorodinitrobenzene (FDNB) has been a topic of great discussion, figures vary between 65% and 100% (Harding 1965). Gustavson (1962) suggested that ϵ-amino groups cannot be involved in cross-linking due to the fact that the shrinkage temperature was not altered by complete acetylation or deamination of collagen. Evidence for the existence of the ϵ-amino peptide link rests almost entirely on the isolation of a tripeptide L,L-N^{ϵ}-(glycyl-α-glutamyl)-lysine from a collagen acid hydrolysate by Mechanic and Levy (1959).

Linkages involving carbohydrate.

Following the finding by Grassmann and Schleich (1935) that glucose and galactose were firmly bound to hide collagen in equal proportions, it was suggested (Grassmann, Endres and Steber, 1954) that the sugars might be bound by ester linkages. Further attempts to remove all the carbohydrate from collagen (Grassmann et al., 1957a, Kuhn, Grassmann and Hofman 1959) showed that 0.5%

hexose was an irremovable part of collagen. Enzymic studies by Hörmann (1960b) showed that the hexoses occurred in the more inaccessible regions of the molecule, suggesting that they take part in cross-links. That the hexoses could be associated with ester links was shown by the concentration of the hydroxylamine sensitive links in these same regions (Hörmann 1960a).

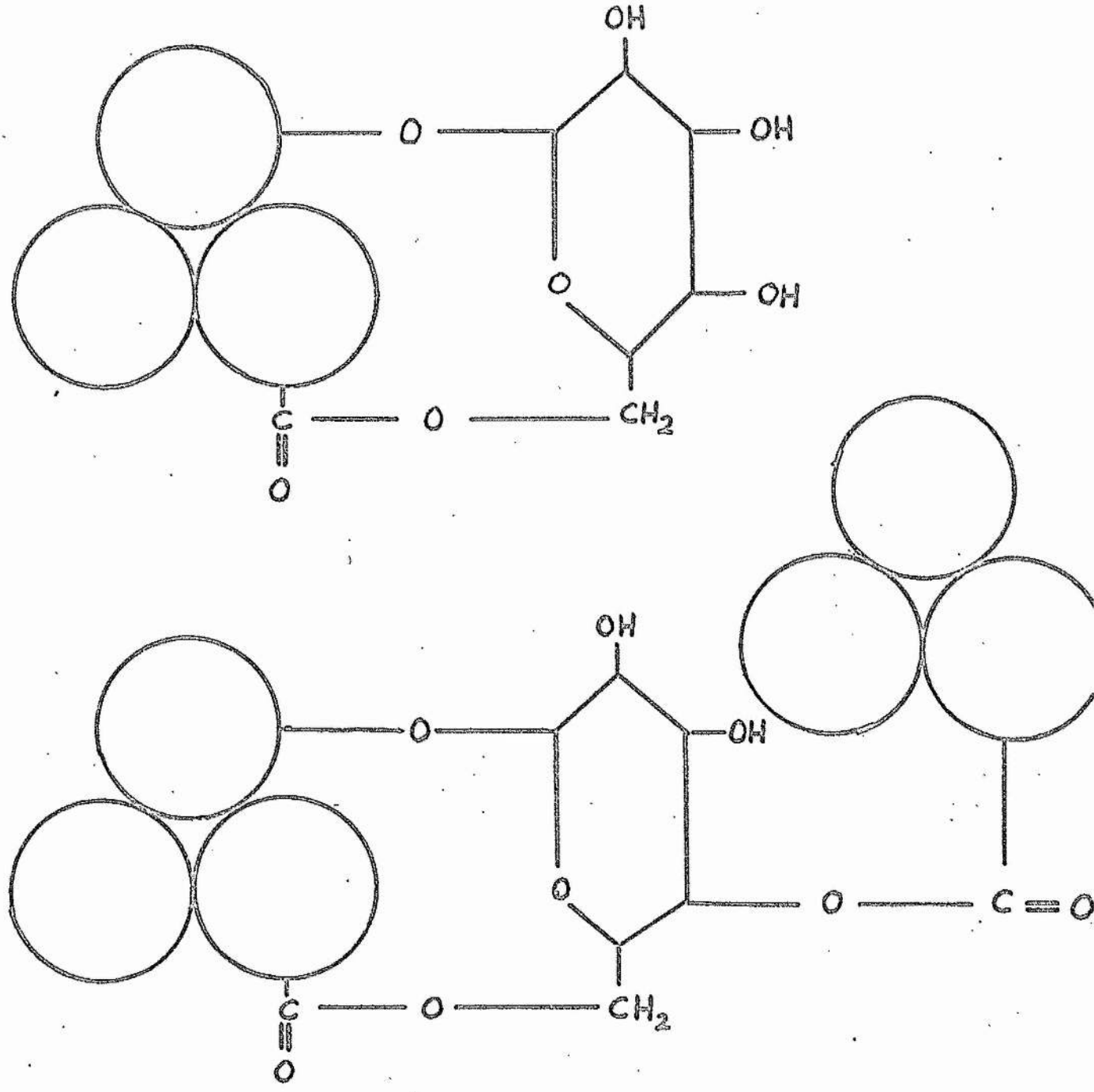
Studies with periodate (Jackson 1954) showed a decrease in the shrinkage temperature after treatment with this chemical suggesting the involvement of hexose with cross linking. It was suggested by Grassmann and Kuhn (1955) that the hexose occurred actually within the chain, due to the breakdown of the chain to peptides with periodic acid, but this was disproved by Hörmann and Fries (1956) using mild periodate oxidation when no amino groups were released, the original conditions must have led to peptide cleavage.

Following results that the breakdown due to periodate in the presence of hydrogen bond breaker was very similar to that due to hydroxylamine plus hydrogen bond breaker (Hörmann 1960b), it was suggested that a rupture of cross links was occurring in both cases, cross links, in fact, involving ester links and hexose.

Hörmann (1960a) has suggested a type of arrangement for the intra and inter molecular cross linking via hexose molecules as shown in Fig. II . The consumption of hydroxylamine and periodate, and an ester/hexose ratio of 2:1 in mature collagen agreed well with this structure (Hörmann 1962).

FIG. II

The type of intra- and inter-molecular cross-linking visualised by Hörmann (1960a).



The type of arrangement visualised in the uncrosslinked collagen by Gallop and his colleagues has already been mentioned, and further studies have shown that during the maturation of collagen from salt soluble to acid soluble there is no increase in the ester links and yet there was a greater proportion of β components (Gallop et al., 1959). This process was visualised as taking place by a process of transesterification (Gallop 1964; Verzar 1963b) see Plate IV . Although inter-molecular as well as intra-molecular cross linking was possible, there was an increased number of ester links in insoluble collagen, probably from some other source and not attached to hexose.

The results of Blumenfeld et al., (1963) do not fit in with the type of crosslinking suggested. They showed that all of the hexose (5 moles glucose plus 7 moles galactose) existed as monosaccharides linked only by carbon 1 to the protein.

Aldehyde cross links

Recent studies by Piez and his colleagues on the splitting of collagen chains with cyanogen bromide and subsequent fractionation of the products have led to the identification of a cross link apparently involving the modified side chain of a lysine residue (Bornstein and Piez 1965 and 1966; Bornstein 1966; Bornstein, Kang and Piez 1966a and 1966b; Piez, Martin, Kang and Bornstein 1966; Piez 1967).

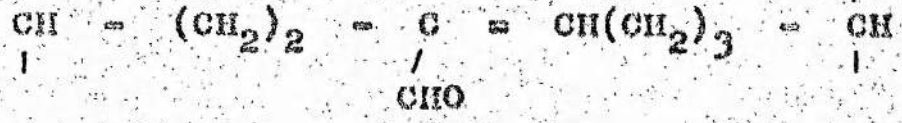
Following their experiments on the fractionation of denatured soluble collagens on columns of carboxyl methyl cellulose into two types of single chains, α_1 and α_2, and

their covalently linked dimers (Piez et al., 1961; Piez, Eigner and Lewis 1963) these authors have simplified their starting materials and adopted a more systematic approach. Pure α_1 and α_2 components have been derived from lathyritic salt extracted rat skin collagen, and the β_{12} dimer from normal acid extracted rat skin collagen. Cyanogen bromide was used to split these chains of the carboxyl terminal side of methionine residues (Gross and Witkop 1961) and the degradation products were fractionated in carboxymethylcellulose and phosphocellulose. It was found that certain small peptides from β_{12} differed in their flow rates in these chromatographic media from those for α_1 plus α_2, although the larger peptide from α_1 plus α_2 and β_{12} were identical in their properties.

Using conditions for cleavage which did not result in breakage of the helical structure of collagen, and also using chymotrypsin and trypsin, Bornstein et al., (1966b) demonstrated that short peptides derived from an amino terminal non-helical sequence of collagen were involved in the cross linking process. These peptides, fifteen residues in length for α_2 and fourteen residues in length for α_1, terminate in a Glycine-Proline-Methionine sequence at the carboxyl end, presumably the start of helical configuration in the molecule (Piez 1967). Furthermore a lysine residue has been shown to occupy the fifth position from the amino terminal end in both types of peptide.

This lysine residue has been shown to occur as an aldehyde function in some peptides and has led to the separation of two types of α_1 and α_2 chains, termed α_1^{lys} and α_2^{lys}, and α_1^{ald} and α_2^{ald}. In labelling experiments it was shown that lysine was a precursor of the aldehyde form. The latter two peptides were absent from cyanogen bromide digests of β_{12} but a peptide present was indicated to be a dimer of the α_1 and α_2 peptides. Lathyritic collagen did not produce an aldehyde containing peptide on fractionation after cyanogen bromide treatment.

The data suggested that a cross link was formed by an aldol type of condensation of two lysyl derived aldehydes on adjacent chains, and that the lysine → aldehyde conversion was blocked in lathyrism (Bornstein and Piez 1966). The cross link was suggested to be of the following structure (Piez 1967).



The fact that this cross linking region was in a non-helical terminal portion of the collagen molecule and that it was split off by the action of chymotrypsin was in good agreement with the work of Schmitt and his colleagues on the telopeptide concept. An aldehyde function has also been described and studied in various tropocollagens by Gallop 1964; Rojkind, et al., 1964 and Gallop, Blumenfeld, Rojkind and Paz 1965. However, the chemical nature and relation to cross linking of this

grouping has yet to be ascertained. It is not inconveivable that this latter aldehyde function is present due to a reaction visualised by Milch (1963 and 1965) of an intermediary metabolite with collagen in vivo.

The effects of association mucopolysaccharides and non-collagenous proteins on the stability of collagen.

Jackson (1952) has shown that there was an increase in the swelling properties and an increase in the solubility of collagen in dilute acetic acid after treatment with hyaluronidase. Further work (Jackson 1953) showed that treatment of collagen with hyaluronidase also lowered the shrinkage temperature by 12°C. These observations were interpreted to suggest the involvement of mucopolysaccharides in the maintenance of fibre stability. Recent studies carried out by Milch (1966) have suggested that the carbohydrate polymers of connective tissue maintain the collagen in a "young" form, by acting as "collagen-plasticising" agents. These compounds were shown to decrease tensile strength and increase swelling and mechanical extensibility of collagen preparations. The results of Milch (1966) would agree, therefore, with some of the suggested mechanisms of aging, where a loss of ground substance during the aging process results in an increase in the stability of the collagen. Mucopolysaccharide has also been implicated as a collagen stabilising factor by Sobel, Gabay, Wright, Lichtenstein and Nelson (1958) and Kao, Hilker and McGavack

(1960). Hörmann (1962) has also suggested that during maturation of collagen, there is an intermediate stage where mucopolysaccharide is involved in a type of non-covalent stabilisation.

Partington and Wood (1963) have presented evidence which suggested that it was more likely that non-collagenous protein constituents of connective tissue had a stabilising action on collagen. Other authors (Harkness and Harkness, 1959; Kuhn and Hannig, 1961) have also suggested that non-collagenous proteins could be implicated in the stability of collagen after studies on the action of trypsin on collagenous tissues and collagen fibres. However the work of Schmitt and his colleagues on the "telopeptide concept" must be considered seriously when interpreting the results of the action of non-collagenase proteases on collagen.

Other types of factor contributing to stability have been suggested. Elden and Boucek (1960) have indicated that salt type links may play an important part. Finally, there is the possibility that water molecules either free (Owsten 1951), or in an ice like cage (Klotz 1958), contribute to tendon stability by encapsulating the fibre (Berendsen 1962).

AGING EFFECTS.

Having discussed the chemistry of the tropocollagen molecule and the morphology of collagen fibres, it is necessary to consider the effects that aging and disease have been shown to have on the properties of collagen. When considering the effects that connective tissue diseases have on collagen and in particular rheumatoid arthritis, which we are specifically concerned with here, it is necessary to take into account the effects of the "normal" process of aging on collagen, for, as is well known the incidence of rheumatoid arthritis is substantially higher in older age groups than in the younger age groups, (Bland and Clark 1963).

All too frequently aging is looked upon as a passive degenerative process, its common association with changes in the transport systems of the body, such as blood vessels, nerves and connective tissue, being well known to any lay person. A study of the processes which eventually take place in any single tissue of the body is to recognise the changes as active processes.

Connective tissue is involved in the mechanism of stress distribution and long term weight bearing functions of the body and thus its involvement in the general aging process of the body is a study which concerns both biochemical and medical centres of research.

In considering sites where the first changes important in aging might occur it would appear likely that the primary reactions related to senescence would take place in molecules

which are static or which have a low rate of turnover rather than in molecules in which constituents are rapidly being replaced. Collagen has a very low turnover rate. (Harkness, Marko, Muir and Neuberger 1954; Neuberger, Perrone and Slack 1951; Neuberger and Slack 1953; Kao, Hilber and McGavack 1961; Thompson and Ballou 1956).

Thompson and Ballou (1956) administered Tritium to pregnant rats and found that the young had labelled tissue at birth and that the half life of the collagen was greater than five hundred days and most of the collagen was not replaced during the animal's life. Neuberger et al., (1951) and Neuberge and Slack (1953) using ^{14}C glycine also showed that the tendon collagen of the adult rat had a half life which was of the same order as the animal's life span. Tendon collagen and skin collagen were rather special in this respect as collagen of liver and bone seemed to turn over relatively fast, however those latter rates of turnover were still very slow compared with the non collagen proteins, (Gerber, Gerber and Altman 1960) Thus it would appear that most collagen is synthesised at an early age, and therefore is susceptible to progressive change with time in the physiological environment in which it exists.

It is worth noting here that collagen may be broken down under certain well defined physiological conditions, and this depends to a great extent on the tissue (Harkness 1961). The collagen of the post-partum uterus has been shown to be quite labile (Harkness and Harkness 1954; Harkness and Moralee

1956) and considerable collagen replacement has been shown to occur in the remodelling of bone (Kao, Hitt, Dawson and McGavack 1962).

Changes in Connective Tissue components with age.

An increase in the total amount of connective tissue in different organs has been considered to be characteristic of aging. Not only the amount but the composition of the connective tissue may undergo some change, (Chvapil and Hruza 1959).

In general, changes in the ground substance amount to an overall decrease in total content. (Sobel, Zutrauen and Marmorston 1953); Houck and Jacob 1958; Kao and McGavack 1959; McGavack and Kao 1960; Kao, Milker and McGavack 1960). Histologically, Gersh (1959) demonstrated diminution of the ground substance in rat tail tendon, skin, thyroid gland and mouse diaphragm as a function of age.

It is apparent, however, that the composition of the ground substance undergoes a change. Meyer, Hoffman and Linker (1958) reported an increase in keratosulphate in human rib cartilage with age while Stidworthy, Masters and Shetlar (1958) reported a decrease of galactosamine (corresponding to a decrease of chondroitin sulphates) and an increase of glucosamine in human rib cartilage with age. Noble, and Boucek (1958) found a decrease in glucosamine and an increase in galactosamine with increasing age in sponge connective tissue. Kao, et al., (1962) showed a decrease in the mucoprotein and an increase in an unidentified glycoprotein in rib cartilage of the rat with increasing age.

Changes in the fibrous portion of the connective tissue involve increase in its total quantity and a consequent increase in the "toughness" of the tissue and in skin, for instance, an increase in a "leathery" appearance. (Gersh and Catchpole 1949; Grant 1963).

In the normal development of the fibrous component of connective tissue it has been found that, in the skin and tendon of animals, there is a decrease in the number of fibroblasts in proportion to the number of collagen fibre bundles from the young to the adult. (Ceresa 1936; Ingelmark 1948; Andrew 1951; Davidson 1963).

Changes involving elastin which occur with increasing age involve a general deterioration of this protein. Calcification occurs on and within the elastic fibre (Lansing, Rosenthal and Alex 1950; Lansing 1959) with a resulting decrease in the elasticity of blood vessel walls and the tissues of the lung (Bowen 1923). However, the amount of elastin appears to remain constant throughout the aging process. (Kraemer and Miller 1953; Lansing and Rosenthal 1950).

Changes in the collagen component of connective tissue during aging have been described by many authors.

Vohwinkel (1931), in studies on the skin of the face, found degenerative changes in the collagen component beginning at about the fourth decade. The changes were found to resemble those caused artificially by X-rays and radium emissions.

Ejiri (1937) also reported histological changes in dermis with increasing age. Hill and Montgomery (1940) believed that those changes were due to exposure since they found no changes in skin taken from the protected parts of the body, e.g. abdomen, in individuals over sixty years old. Goldzieher (1946) noticed general degenerative changes in the collagen from skin of aged individuals. These changes involved hyalinisation, fragmentation and a reduction in the number of nuclei.

Bucciante and Lauria (1934) indicated an increase in the percentage of collagen in the striated, non cardiac, muscle with an increase in age. Myers and Lang (1946) found, by chemical analysis, an increase in the percentage of collagen, after the age of fifty, in the thoracic aorta. Harman and Webster (1949) found an increase in the proportion of collagen from the atria of some individuals over the age of ten. However no increase was found in the ventricles.

Gross (1950a) and Randall, et al., (1952) demonstrated a marked increase in the proportion of collagen in the skin of rats between 20-35 days after birth and in the tail tendon of rats 16 days after birth.

Further studies listed below have also shown increases in the collagen content of the animal tissues mentioned.

The skin of the rat and that of the human being. (Sobel, Zutrauen and Marmorston 1953; Houck and Jacob 1958; Kao and McGavack 1959; McGavack and Kao 1960; Kao et al., 1960; Sobel, Gabay, Wright, Lichtenstein and Nelson 1958).

The femur of the rat. (Sobel, Marmorston and Moore 1954).

The human lung. (Briscoe, Loring and McClemont 1959).

The muscle, heart and liver of the chick embryo. (Herrmann and Barry 1955).

The aorta, tendon and uterus of the rat. (Kao and McGavack 1959; McGavack and Kao 1960; Kao et al., 1960).

The intima of the coronary artery. (Moon and Kinehart 1952).

Associated with the increases in the fibrous nature of connective tissue with age are observations on the increase in tensile strength of tissue, in particular, skin. Mendoza and Milch (1964, 1965) and Fry, Harkness and Harkness (1964) have shown an increase in tensile strength of goat and rat skin, which was related significantly to the age of the animal. Nimni, de Guia and Bavetta (1966) have found very good correlation between tensile strength, insoluble collagen content and age in rabbit skins. As might be expected the increase in insoluble collagen content was very closely paralleled by the tensile strength.

These studies on age changes in connective tissues all indicate that there is an overall decrease in the ground substance demonstrable by a decrease in the mucopolysaccharide content and an increase in the fibrous nature of the tissue, due to an overall increase in collagen content. The ratio of Hexosamine to

Hydroxyproline gives a good indication of the proportion of mucopolysaccharide to collagen, or ground substance to fibrous component.

This ratio is now generally accepted as a measure of biochemical age (Sobel and Marmorston 1956; Sobel et al., 1958; Clausen 1962; Kao et al., 1960). A decrease in the ratio occurs with advancing age and this has been shown to occur in the tissues of rats, guinea pigs, rabbits and man (Sobel and Marmorston 1956; Sobel et al., 1958).

In a recent study by Anderson (1965) on the effects of radiation on the connective tissue in Hiroshima bomb survivors, a decrease in the ratio has indicated a premature aging of these individuals. This accelerated aging, as indicated by a decrease in the ratio, due to radiation effects has also been shown to occur in the tissues of rats when exposed to X-rays (Sobel, Gabay and Bonarris 1960).

Associated with the increase in collagen content of connective tissue with advancing age is an increase in collagen fibre size.

Gross (1950a) in an electron microscope study of aging rat skin demonstrated that besides a decrease in the amorphous ground substance with aging there was an increase in the average diameter of the collagen fibre bundles. He also found similar changes in human aged and young skin (Gross 1950b and 1950c). Von Pahlke (1954) observed these changes in human achilles tendon, Schwarz (1953, 1957) in human sclera and in a variety of

animal tissues. It was interesting that the changes became stabilized at the age of 60 days in rat skin, 13 years in achilles tendon and after 7-8 months of foetal development in the human sclera. Porter (1951) observed an increase of collagen fibril width in progressively older tissue cultures of fibroblasts.

It has also been suggested that there could be an increase in the length of the collagen fibril. This suggestion was put forward after it was shown that the collagen fibril could be seen to have smoothly tapered ends under the electron microscope. Porter and Vanamee (1949) described these tapered ends in cultures of fibroblasts and Randall et al., (1952) noted a large number of tapered ends in the umbilical cord of a ten day old embryo rat. Banfield (1952b) showed that shorter fibres were abundant in embryos but scarce in adult skin.

Randall et al., (1952) and Porter and Vanamee (1949) have described collagen fibrils which had a periodicity of 270Å. This was suggested to be characteristic of an immature collagen, the native type periodicity of 640Å gradually appearing as the age of the tissue culture increased. It seems unlikely that this is an immature collagen and could possibly be a reticulin which does show a periodicity of 210Å.

The effect of age on the properties of collagen.

The overall increase in quantity and size of collagen fibres in connective tissue during the process of aging is accompanied by a change in the collagen protein itself as indicated by various studies on collagen solubility, swelling, enzyme susceptibility, X-ray diffraction and shrinkage temperature

It is well known that only a proportion of the collagen present in connective tissue is soluble in aqueous solvents of a neutral pH or even of a pH approaching 3 or 2. This type of observation, where a polymer or a protein is only partially soluble in a solvent which causes no chemical or physical degradation of the substance, has been said to be indicative of a cross linked system.

Collagen, in fact, is often characterised by its extractability as neutral salt soluble collagen, acid soluble collagen or insoluble collagen. The use of 0.14M sodium chloride, 0.45M sodium chloride, 1M sodium chloride and 2M sodium chloride as solvents removed progressively more elements of collagen from a tissue (Jackson and Bentley 1960). Furthermore, the solubility of collagen, in the more dilute solvents, decreased as the age of the animal source increased. This latter observation was first made by Nageotte and Guyon in 1934 and has been confirmed by many authors since that date using a variety of animal tissues. For instance, human collagen has been shown to become more insoluble with age of the individual by Banfield (1952a) and Gross (1959), the relative amount of acid soluble collagen being maximal in huma

skin a few weeks before birth and decreasing exponentially throughout the life span, that is to the age of sixty (Banfield 1952a; Kohn and Rollerson 1959b; Bakerman 1962; Harkness 1961). The solubility difference in skin with age is much greater than that of tendon. Verzar and Meyer (1961) showed an overall decrease in the soluble collagen and an increase of insoluble collagen in the tail tendon of rats of increasing age, and Aslan and Vrabiesco (1965) showed an increase in the acid soluble fraction and a decrease of the neutral salt soluble fraction with age in the same tissue. Cadavid, Denduchis and Mancini (1963) showed a decrease in soluble collagen with age in rat skin. The extractability of human abdominal skin collagen in 0.1% acetic acid showed an overall decrease with age (Banfield 1959; Banfield and Brindley 1959). The extractability of human skin collagen in 0.1M borate buffer pH 8.7 has been shown to decrease with age to a constant level in the sixth decade (Hall 1956).

It is interesting to note here that, with reference to the work of Hill and Montgomery (1940) on the effects of exposure, Smith, Davidson, Sams and Clark (1962), while demonstrating a decrease in soluble collagen and an increase in insoluble collagen in skin with age, also noted a reversal of this process in skin which had suffered from over exposure to the sun.

Mills and Bavetta (1966) have shown that rat bone contains a relatively large soluble collagen fraction at birth, this fraction decreasing and the insoluble fraction increasing with increase in age.

Rao and McGavack (1959), McGavack and Rao (1960), Hutterer, Rubin, Singer and Popper (1959) and Carmichael (1966) have all observed an increase in the insoluble fraction of collagen with increasing age of the tissue source.

Schaub (1963) has found a decrease in soluble collagen from birth to old age in all tissues, but in the parenchymatous organs, liver, lung and kidney, the decrease was more rapid.

A decrease of solubility of collagen was included in a series of results which indicated a rather more general decrease in solubility of several proteins during the aging process, (Medvedeva 1939; Dische, Borenfreund and Zelmenis 1956; Benetato, Opreanu and Musteanu 1939).

Jackson (1958) indicated that during the biogenesis of the collagen fibril, the fibroblast could be first assumed to synthesise neutral salt soluble collagen which was then converted to citrate soluble collagen and finally into insoluble collagen. This process could take place in the manner visualised by Fitton-Jackson (1953) that the procollagen is added to the preformed matrix thus forming a thicker fibril with further growth.

Harkness, Marko, Muir and Neuberger (1954), Jackson (1957a and Green and Lowther (1959) have all suggested that dilute salt solutions extract freshly produced collagen. In fact, that fraction of collagen removed with 0.15 - 0.45% sodium chloride is generally accepted to be newly synthesised material. According to Gross (1958) this fraction essentially disappeared when a tissue stopped growing; however, that fraction extractable in acetate/

acetic acid buffer at pH 3.5 and below, or at higher ionic strength (e.g. 1M sodium chloride) was found throughout the life of the animal.

Thus from the majority of studies on the different forms of collagen occurring during maturation of connective tissue, it is apparent that, in general, neutral salt soluble collagens represent the first formed collagen and this matures into a type of collagen which is soluble in increasingly stronger salt solutions. This type is then converted to an acid soluble collagen and finally an insoluble fraction emerges. However Kuhn (1963) has shown by ^{14}C-glycine incorporation that the neutral salt-soluble fraction is converted directly into insoluble collagen in the skins of rats and he suggested that acid soluble collagen was not a true precursor of the insoluble substance. Nimni, de Guia and Bavetta (1965) have also indicated that citrate soluble collagen is not an immediate precursor of insoluble collagen in rabbit skin.

From these experiments it would appear that intra and inter-molecular stabilising cross-linkages were formed simultaneously. Kuhn has found that the formation of both types of linkage was blocked to the same extent in lathyritic rats.

Studies by Kawai, Kimura, Miyata and Nishihara (1964) have suggested a structural difference between neutral salt soluble collagen and acid soluble collagen. This difference is quoted as being of the order of 35,000 in molecular weight, the acid soluble

collagen molecule being larger by this amount and also 300Å longer than the neutral salt soluble collagen molecule. Furthermore, denaturation and fractionation of the two types led to their observation that there was complete absence of the γ component in the acid soluble fractionation, and no β component in the neutral salt soluble fractionation. They seemed to infer that the acid soluble fraction was not a direct precursor of the insoluble fraction but that the γ component in the salt soluble fraction was this precursor.

Changes of the swelling capacity with age.

Banfield (1952a, 1956) and Kohn and Rollerson (1958, 1959a) have shown that there is a decrease in the swelling of human tendon collagen in acetic acid and some alkaline solutions associated with increases in age. These changes are again indicative of an increase in the stability of collagen with increases in age. Studies of Bowes and Kenten (1950) are significant in that they postulated a breakage of some intermolecular bonds (hydrogen bonds or amide bonds) on swelling in alkaline solutions and thus an increase in the number of such bonds would inhibit swelling.

The results of Kohn and Rollerson (1958) are interesting in that they showed a rapid decrease in swelling ability over the ages of 30 to 50 but a very gradual decline at other ages. This may be of interest with regard to the theory of Bidder (1932) who viewed aging as beginning after an animal had reached a specific genetically determined age, generally indicated by the age at which its offspring had become independent.

Changes in the susceptibility to collagenase with increasing age.

Keech (1954, 1955) suggested that there was a decrease in the susceptibility of collagen from human skin to collagenases with an increase in age of the tissue source. Although the results presented were not really significant there was a stimulus for further work and Kohn and Rollerson in 1960 showed more convincingly that young collagen was more digestible by collagenases than old collagen. These latter authors also demonstrated a decreased susceptibility to collagenases after formaldehyde tanning of the collagen and they concluded that a chemical reaction, possibly of the tanning type, could be involved in the aging process.

Woessner (1962) has also demonstrated an increase in the stability of human collagen to collagenase with age.

Changes in the X-ray diffraction pattern with age.

X-ray diffraction studies have shown that the degree of orientation in a collagen fibril increased with age (Kratky, Lauer, Ratzenhofer and Sekova, 1962), and that there was a microscopically observable improved orientation of connective tissue as a whole (Joseph and Rose 1962). Feitelberg and Kaunitz (1949) in X-ray studies on human chordae tendinae observed poor orientation of the collagen fibres under the age of four years and increasingly improved orientation up to the age of 45, above this age all specimens were well orientated.

These changes might suggest an increasing stability of the collagen fibre with advancing age, and, in fact, Clark and Zieglor (1936) did correlate an increase in tensile strength of cat gut

ligatures with an improved orientation using X-ray diffraction techniques.

The effect of age on the shrinkage temperature of collagen and contraction in lyotropic solvents.

The effect of age on the shrinkage temperature of collagen isolated from different sources has been well documented (see, for example, Verzar 1964; Gustavson 1956) Verzar, in fact, was the first to demonstrate that thermic contraction and relaxation of collagen fibres depended on the age of the animal (Verzar 1955 a,b, 1956a,b). He has demonstrated that the load required to prevent thermal contraction of rat tail tendon varied directly with the age of the animal (Verzar 1956a,b, 1957a,b, Brocas and Verzar 1961a).

An increase of the temperature at which shrinkage occurred, associated with increasing age, has also been demonstrated for collagen from:-

Human sources (Brown and Consdon 1958, Brown, Consdon and Glynn 1958,).

Rabbit skin (Verzar and Huber 1958,).

Rat skin and tail tendon (Bjorksten, Andrews, Bailey and Trenk 1960, Chvapil and Jonsovsky 1963, Radhakrishnan, Ramanathan and Nayudamma 1964).

The African clawed toad, Xenopus laevis, (Brocas and Verzar 1961b).

It has also been found that the extent of contraction of collagen fibres in lyotropic solvents increased with age. Banga, Balo and Szabo (1954, 1956) used 40% potassium iodide and acetic

acid as two types of solvent. Elden and Doucek (1959) used 0.5M acetic acid and Chvapil and Hruza (1959) and Chvapil and Jensovsky (1963) used 2.5M sodium perchlorate. These agents were visualised as acting by direct competition with the valency forces and therefore interfering with the cross-links.

The contraction of the collagen fibre due to heat or lyotropic solvents is generally considered to result from the weakening or dislocation of the inter-chain crosslinks of the collagen fibrils (Gustavson 1956). Thus, any increase in the temperature needed to shrink a specimen of collagen suggests a greater degree of cross linking in that sample. A decrease in the extent of contraction in lyotropic solvents implies the same process.

Further effects of aging on the properties of collagen, from more recent studies.

Further, more direct evidence, for an increase in cross linking was presented by Piez and his colleagues with the fractionation of various types of soluble collagens on carboxymethylcellulose and also by Kulonen and his colleagues with the fractionation of soluble collagens on starch gels and use of the 'Flory equation'. The results of these authors will be briefly discussed.

Fractionation of denatured soluble collagen with respect to age effects.

The work of Piez and his colleagues on the fractionation of denatured soluble collagen, already discussed, has led to the assumption that an increase in the β component and the γ component occurred during aging due to an increase in the

cross linking. Piez, Eigner and Lewis (1963) showed an increase in β_{11} and β_{12} fractions in collagen from mature rat skins compared with the younger rats. Bornstein and Piez (1966) showed that α chains of salt extracted collagen (assumed to be recently synthesised) contained a hydroxylysine residue which appeared to occur as an aldehyde function in some α chains of acid extracted (more mature) collagen. This was comparatively indirect evidence for the cross linking of collagen to form dimers and trimers of α chains, during aging, of the type already mentioned (Page 45).

More direct evidence has been supplied recently by Kulonen and his co-workers. These workers have fractionated collagen from the tail tendon fibres of rats of various ages using starch gel electrophoresis as the separating method, (and also have estimated the cross links present in collagen from the skin and tail tendons of rats of various ages). Kulonen, Mikkonen and Heikkinen (1963) and Heikkinen and Kulonen (1964) demonstrated that, in older rats, collagen from the skin showed a decrease in the α fractions and an increase in the β fractions plus the appearance of a slow moving component, (possibly γ).

These results are evidence for an increase in the intra-molecular cross linking; the isolation of the β_{22} component by Bornstein, Martin and Piez (1964) and Bornstein and Piez (1964) from guanidine extracts of human skin was evidence for a form of inter-molecular cross linking.

Aslan and Vrabiesco (1965) have recently demonstrated that there was an increase in the specific viscosity of soluble collagen with age, thus indicating a component of greater rigidity, such as was visualised by Pioz and his colleagues, to occur by intra-molecular cross linking. The possibility of a polymeric form of tropocollagen stabilised by inter-molecular cross links, existing in solution and thus leading to a rise in specific viscosity, cannot be dismissed.

Evidence for increased cross linking using the Flory Equation

Direct evidence for the increased formation of inter molecular cross-links during aging is scarce although the process has been suggested many times (see previous references). However, some authors have used a method developed by Wiederhorn and his colleagues which can estimate the number of cross links in denatured collagen. The method is based on the statistical elasticity theory of Flory and Rehner (1943) (see also Flory 1953 Wiederhorn and Reardon (1952) and Wiederhorn, Reardon and Browne (1953) showed that when thermally contracted collagen was swollen in appropriate media it obeyed rubber elasticity theory. Using the equation below it was possible to determine the average molecular weight between junction points (cross linking sites).

$$f = \frac{RT\rho\, V^{1/3}}{M_c} \left(\alpha - \frac{1}{\alpha^2}\right)$$

where f = force in g./cm^2. of the cross sectional area of the non-extended fibre.

R = gas constant

T = absolute temperature

V = volume fraction of dry sample in swollen sample.

ρ = density of dry material.

α = ratio of extended fibre to non-extended fibre.

M_c = average molecular weight between junction points.

Kulonen, Mikkonen and Heikkinen (1963) and Heikkinen, Mikkonen and Kulonen (1964) have used this method to determine the number of cross links in samples of collagen from the skins and tails of rats of different ages. In tail tendon collagen they found a value for M_c of 50,000 - 65,000 which did not change with advancing age. However, for rat skin collagen the following values were obtained.

Age	Average M_c
3 months	213,000
6 months	99,500
12 months	49,800
24 months	40,000

These indicated an increased cross linking and the authors further suggested a system of cross links of varying strength similar to that proposed by Verzar (1963c).

Adam, Bartl, Deyl and Rosmus (1965) and Adam, Deyl and Rosmus (1966) have used the method to show that gold salts will cross link collagen in vivo; they have expressed the results as

moles of cross link/cm^3. They found an increase in apparent cross linking associated with an increase in shrinkage temperatur The method has also been applied by Bowes and Cater (1964) to the assessment of cross links introduced into kangaroo tail tendon by tanning agents. Those authors make the statements; (a) that for the derivation of the formula it was apparent that two cross linked chain segments, M_c, must be involved in each cross link, and hence there is one cross link per molecular weight unit $2M_c$; (b) that the method only measured cross links in excess of those necessary to give a continuous network in the first place.

Results quoted by the authors cited showed that the method may show an increased cross linking during aging and during reaction with cross linking agents, the provisos suggested by Bowes and Cater must, however, be borne in mind.

Theories Suggested for The Aging Process.

Verzar (1958) quoted the various theories of the aging process which have led up to the present day hypotheses. An early theory taught by Metchnikoff was that aging changes were due to intoxication by intestinal bacteria. Brown-Séquard in this century was interested in the effect of injections of sex hormones on age changes, and even carried out experiments on himself. In 1954 Bogomoletz was putting forward his hypothesis of a serum which destroyed reticulocytes; these cells he believed were deleterious to connective tissue, in particular to collagen. His work was published as a book but has little acceptance at the present time.

The implication of the association of increased cross linking with aging has caused a great deal of speculation on the methods by which cross links could be introduced into the collagen molecule in vivo. That aging of connective tissue is due to a change in the content of cross links in the tissue was anticipated in what, at one time, was known as the colloidal theory of aging. Ruzika (1924) made the statement that senescence is analogous to the gradual coagulation of colloid systems. Gortner and Gortner in their textbook of Biochemistry (1949) make the statement "If the problems of rejuvenescence were ever solved they would be solved largely through colloid chemical studies designed to bring about an increased, or to maintain, an imbibition capacity of the tissue colloids."

It is now accepted that, rather than a type of tissue coagulation, an actual cross linking of the collagen molecule occurs which causes the observed changes.

(1) Aging as an in vivo tanning process.

Bjorksten and Champion (1942) suggested that the increasing rigidity of tissues in the body as a result of aging might be due to a tanning process caused by the content of aldehyde in the blood. Ri (1940) had shown that the aldehyde content of human blood was very high, so high in fact that in the light of experience with industrial protein gels and the tanning of skins to form leather, it should amply suffice to tan the body proteins to complete rigidity and loss of elasticity within about two months.

Bjorksten and Champion suggested that the conditions of repeated stretch and relaxation which are present in the body tissues leads to an orientation of the tanning bridges, which do form, in such a way that they do not interfere with the stretching and relaxation of the tissue. They further suggest this as a reason for the beneficial effects of exercise and the atrophy of organs under conditions of continued immobility.

Bjorksten (1958, 1962) further expanded his theory suggesting the formation of irreversible cross linkages between various nutrient moieties and constituents of connective tissue. He demonstrated the formation of non-utilitarian compounds in vitro through binding of protein, carbohydrate and lipid materia

Milch and Murray (1962) and Milch, Murray and Kenmore (1962) using the thermal shrinkage of collagen as an indication of cross linking density showed that glycoraldehyde, the major intermediary through which carbohydrate metabolic pathways must pass, is a tanning agent (cross linking agent) for collagen 'in vitro' under conditions approximating those of extracellular body fluids. Milch (1963) showed that a few water soluble aliphatic aldehydes of metabolic origin were capable of cross linking polypeptide chains of the collagen type. He noted that the resulting molecules appeared to have many of the known properties of collagen molecules in aged and aging connective tissues. In 1965 Milch further suggested that aldehydes reacting with collagen would do so only with the side chains of lysine, hydroxylysine, arginine, asparagine and glutamine.

It is worth bearing in mind that cross linking is the only process known by which a single small molecule can drastically change the behaviour of two macromolecules of the same type. (Bjorksten 1963).

Other authors who have proposed that covalent bond formation similar to a tanning reaction might produce cross links in aging collagenous structures include, Elden (1959), Gallop and Seifter (1962) and Veis and Drake (1963).

(2) <u>Aging as a free radical reaction.</u>

Harman (1956) suggested that the changes which occur due

to irradiation and those which arise spontaneously in the living cell and connective tissues may have a common source, the OH and HO_2 radicals. These arise on the one hand by the dissociation of water and on the other largely due to the interaction of the oxidative enzymes with oxygen and hydrogen peroxide, with probabl involvement of such trace elements as iron, cobalt and manganese. He suggested further that the maintenance of a high concentration of easily reducing substances, such as cysteine, might be expected to slow down the aging process.

(3) Aging due to chemical and physical changes in irreplacable macro-molecules.

Sinex (1957) suggested that, due to the low turnover rate of collagen, (Neuberger et al., 1951; Neuberger and Slack 1953; Thompson and Ballou 1956) the human being might face the problem of maintaining the collagen in their connective tissues in a native state for over half a century of continuous incubation at 37 - 38°C, and thus altered molecules might accumulate.

Using a theory of absolute reaction rates he suggested that collagen may undergo a change due to thermal denaturation at 37 - 38°C similar in nature to the change suggested by Pankhurst (1947), and termed "incipient shrinkage". Sinex believed that, of all agents capable of altering macro-molecules, probably the most important was heat, the thermal energy of the atoms vibratin and twisting and stretching chemical bonds and exerting a steady influence for the alteration of structure. According to the theory of absolute reaction rate the denaturation of a macro-molecule requires a preliminary activating step which involves an

appreciable increase in heat content and entropy. Once the molecule enters the activated state denaturation may proceed spontaneously. From Weir's (1949) experiments on kangaroo tail tendon the time for half shrinkage can be calculated at different temperatures. Those times are listed below -

215 years at 30°C

1.92 days at 53°C

4.32 minutes at 63°C

This demonstrates that continuous deterioration might be going on at body temperature.

Sinex (1960) has further attempted to determine the release of amide nitrogen from collagen at 38°C. He suggests that as the amide nitrogen is released at 100°C (pH7) to the extent of 21%, then at 38°C it would take 15 years for 21% release and this would obviously alter the properties of the collagen. However he did not find it possible to evaluate the stability of amide groups at 38°C although there was an increase in the release of amide nitrogen at the shrinkage temperature presumably due to exposure of amide groups which were normally sterically blocked in the native state.

These time dependent chemical changes could therefore be of a variety of types and could include thermal denaturation involving unfolding of the tertiary and quaternary structure (Sinex 1957; Johnson, Eyring and Polissar 1954), a process of oxidation possibly similar to that mentioned by Harman (Sinex 1959) and the hydrolysis of amide, or even peptide, groups (Sinex 1960).

The Types of Cross links suggested to increase in Aging

Of the types of cross link found in collagen, various authors have suggested an increase in their quantity when passing from neutral salt soluble collagens to the, generally accepted, more mature acid soluble and insoluble types of collagen.

Bollo (1960) in his studies on ester links and their reaction with hydroxylamine suggested that there was an increase in the ester type of cross link with increasing age. Joseph and Bose (1962) have shown an increased fixation of hydroxylamine with increase in age of the collagen indicating an increase in ester like cross links. The results of Hörmann (1960a) indicated an increase in ester linking during the conversion of procollagen to acid soluble collagen, although Gallop (1964) did not show such an increase but visualised a transesterification, intra-chain → inter-chain cross link and intra-molecular → inter-molecular cross link. The increased esters found in insoluble collagen are probably from other sources. Joseph and Bose (1962) have demonstrated in rat collagen that the availability of arginine to destruction by hypobromite decreased from 88% to 65% during the lifetime of the animal, (about two years).

Joseph and Bose (1959) and Deasy (1956) suggested that γ-glutamyl peptide links might occur in the chain; however, Joseph and Bose (1960, 1962) found that there was an increase in the succinic acid liberated by oxidation of γ-glutamyl residues after enzyme treatment of aging collagen. Since it would not be expected that this type of residue would increase in the

peptide chain they supposed that there was an increase in the cross links formed via glutamyl γ-carboxyls during aging.

Further studies by Joseph and Bose (1962) showed that the availability of the ε amino groups of lysine to FDNB decreased with age in rats, and they have also demonstrated an increase in bound carbohydrate.

On the basis of the reaction of trypsin on heat denatured mature hide collagen (Joseph and Bose 1960), Joseph and Bose (1962) suggested that a certain proportion of the side chains of lysine and arginine residues in collagen are involved in some type of cross link. They found that only 67% of the arginine and 72% of the lysine linkages were ruptured and suggested that this indicated that the lysine and arginine residues involved in the stable links must be <u>modified</u> in some way, that is, they do not conform to the specificity type, which has been discussed by Bergmann and Fruton (1941). In other words they are modified in some way so that the adjacent peptide bonds are not in the environment necessary for trypsin action. This decrease in availability was shown to be a function of the age of the collagen (Joseph and Bose 1962). However these results could also be explained by an increased masking of the side chain groups due to other factors.

If cross linking occurs by a reaction with aldehydes (Milch 1963, 1965) then it would seem that there should be an increase in these functions during aging. However, evidence for such an increase is not forthcoming although Piez and his colleagues (Piez <u>et al.</u>, 1966) have indicated that an aldehyde

function was formed by conversion of the ε-amino group of a lysine residue prior to the cross linking which appeared to occur during maturation.

The general aging process, though still not completely understood, has been summarised by Grant (1963) and suggested to be the result of three processes:-

1). The gradual accumulation of harmful substances, (the ashes of metabolic fires), and of injuries produced by the business of living, particularly by penetrating radiation such as cosmic rays or gamma rays.

2). The gradual loss of certain vital materials which are consumed somewhat more rapidly than they are replaced.

3). The slow physical changes of the body proteins from a "plump, water-rich condition typical of youth", to a "dried out, leathery state typical of old age". Why the loss of water occurs is not known, but it may be connected with the cessation of growth. In fact it may be generally correct to say that when growth ceases senescence begins and that the more slowly an animal grows the longer it will live. The latter statement is borne out by the work of McCay on the effects of undernutrition on longevity, where an apparent decrease of aging was found (McCay 1958).

Summing up the chemistry of collagen and the effects of aging on the chemistry of collagen and the morphology of collagen fibres it is apparent that the whole system is very complex. Many contradictory results exist in the published literature and many conclusions are drawn from scanty evidence. However, it would seem reasonably well established that the tropocollagen molecule exists in the form of a triple helix and that there is a

type of "orderliness" in the amino acid sequence of the individual poly-peptide chains. That stability is conferred on the molecule by a type of cross-linking, both of an intra-molecular and inter-molecular type, is also reasonably well established, and this process may also be safely inferred to increase during the process of aging. However, from the vast amount of published work on the form and number of these cross-links it is difficult to conclude which particular type, or types, if any, contribute most to the process of maturation.

CONNECTIVE TISSUE DISORDERS.

IN PARTICULAR RHEUMATOID ARTHRITIS.

The Concept of Connective Tissue Disorders.

Up until 1923 connective tissue was considered to have a static and purely structural role due, in part, to the attitude of nineteenth century anatomists, in particular Virchow, who, while observing the intercellular substance, regarded it as having no functional significance other than to act as a support for the cells, which were, according to him, responsible for all bodily activities.

In 1923 Schade introduced the concept of the "connective tissue organ". He realised that this was concerned with water metabolism and ionic equilibria.

The concept that there are primary disorders of the connective tissue is also fairly new. The individual conditions now grouped together under the collective term 'connective tissue disorders' have been recognised for some time; they include rheumatic fever, rheumatoid arthritis, systemic lupus erythematosu polyarteritis nodosa, scleroderma (progressive systemic sclerosis) and dermatomyositis.

The earliest histological change found to be common to these disorders was fibrinoid degeneration. This was observed as early as 1880 by Neumann and mentioned in 1933 by Klinge as the most significant pathological finding in hypersensitivity states.

Klemperer, Pollack and Baehr (1942) realised that fibrinoid degeneration could occur in conditions other than hypersensitivity states and also appreciated that it was a condition seen in the diseases which they considered to be due to

disorders of the connective tissue.

They advanced the view that connective tissue could be the primary site of disease (Klemperer et al., 1942) and further pointed out that the connective tissue throughout the body was affected in systemic lupus erythematosus and scleroderma, as in rheumatic fever. However, the point was made that although each of these diseases had a common basis in connective tissue this "was not to be taken to mean that they had a common aetiology or were even related aetiologically".

With the introduction of disorders of connective tissue was also introduced the term 'collagen diseases'. This term has been much abused and in fact has given way to that of "connective tissue diseases" as it has been recognised for some years that collagen is not always solely affected. Robb-Smith (1960) has defended the term 'collagen disease' and there is much truth in statement made by Klemperer (1950) "When further basic research has clarified the factors which control the plasticity of the connective tissues under normal and abnormal conditions, the concept of collagen diseases will have well served its purpose".

The terms Arthritis and Rheumatism are sometimes used synonymously. In a stricter sense arthritis describes inflammatory disease of articular structures and rheumatism, a more generic expression, includes all pathology of the joints, tendons, ligaments muscle and supporting connective tissue.

There are two major types of arthritis, osteo-arthritis and rheumatoid arthritis. These were separated definitively in 1909 by Nicholls and Richardson. They are entirely different in their mechanism, osteo-arthritis being a primary degeneration of cartilage, rheumatoid arthritis being a primary inflammation of the synovium which may lead to more widespread involvement of non-articular processes.

The lesions which involve the synovia and capsules of the joints are responsible for the classical clinical picture of rheumatoid arthritis. The synovial lesions have been extensively investigated by biopsy and show oedema and mononuclear cell infiltration with a deposition of fibrin in the early stages. Later hypertrophy of the lining cells and accumulation of lymphocytes lead to the formation of a vascular pannus which in its turn becomes fibrosed into a thick membrane over the cartilage. The cartilage subsequently becomes destroyed and the bone immediately beneath it contains proliferating connective tissue. The circumstances are thus produced which can lead to fibrous or even bony ankylosis.

The General Prevalence and Relative Incidence of the Rheumatic Diseases.

A health interview survey carried out in the United States between July 1963 and June 1964 indicated that about 12,000,000 of the civilian non-institutional population of the United States suffer from some sort of rheumatism. It has been further stated (Edwards 1964) that Rheumatoid Disease accounts for a loss of more than 115 x 10^6 work days per year resulting in an annual loss of approximately £500 x 10^6.

In Great Britain similar but less widespread surveys have been carried out, for example by Anderson and Duthie (1963) where in a sample of 1,422 dock yard employees in Scotland 37% experienced rheumatic complaints. A survey of a coal mining population near Edinburgh by Anderson, Duthie and Moody (1962) showed that 44% of 970 interviewed had rheumatic complaints during the previous year, rheumatism in fact accounted for 15% of all sickness absence.

Population surveys by Kellgren, Lawrence and Aitken-Swan (1953) and Lawrence (1961) have shown that the frequency of rheumatic complaints increased from 7% in the youngest age group (15 - 19) to 40% of those over 40. Kellgren (1962) has listed a table of diagnostic criteria for population studies in rheumatoid arthritis, and it is apparent that this condition is uncommon in young people up to the age of thirty (1 - 3%) but that the prevalence increases with age so that after the age of 65 5% of men and 10% of women are affected. Rheumatoid

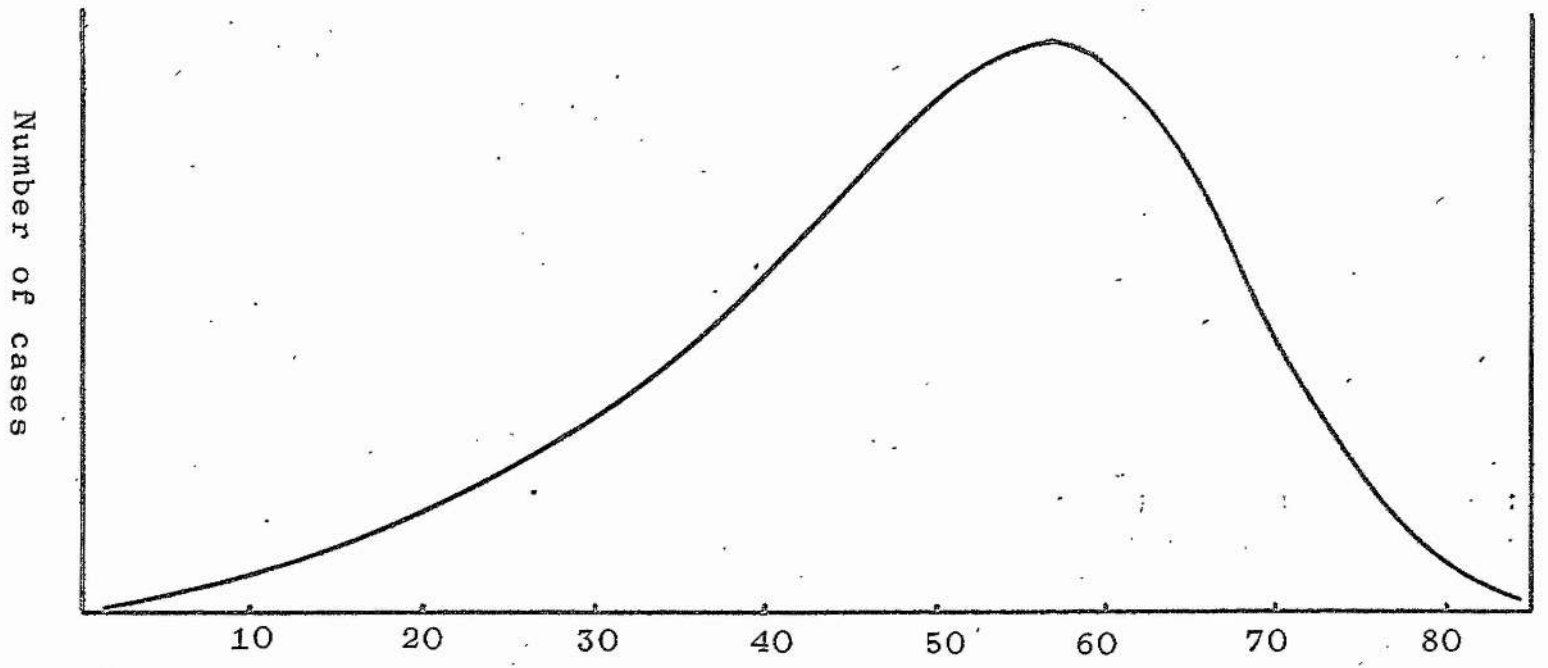


FIG. III

The age distribution of collagen diseases.

(Bland and Clark, 1963)

Arthritis also seems to run in families, in most studies it has been encountered about two to four times more frequently in the blood relatives of cases than in the general population, although this is not a unanimous opinion.

Summing up the social and economic aspects of this disease an estimated 1,700,000 people have rheumatoid arthritis in Britain, at least 1% of all males and 3% of all females (Bywaters 1963). All social classes are equally affected, and the disease has not been shown to be associated with any particular occupation although in some occupations its presence does affect the efficiency of work and hence leads to absenteeism out of proportion to its incidence. With osteoarthritis, on the other hand, occupation is closely related to incidence, jobs requiring heavy lifting lead to wearing out of discs in the lumbar spine and joints in knees. Cotton workers suffer from osteoarthritis in the finger joints.

Until fairly recently it was thought that people living in primitive conditions or tropical climates might be immune, records indicated that it was a disease present in all developed communities living in temperate climates. However, typical cases have been reported among natives living in both West and East coasts of tropical Africa and among Jamaicans, American Indians and Eskimoes (Kellgren 1965). Although there is little information from India and China, it seems likely that this disease will be found to occur throughout the world, though, curiously enough, one immune population does exist, that of the island of Tristan da Cunha.

The Effects of Diseases of Connective Tissue on the Tissue as a Whole

Many of the diseases of aging persons, such as various forms of arthritis, vascular and perivascular diseases and many other ailments affect, or are due to, alterations in connective tissue or in its several functions.

Unna as early as 1896 (quoted by Sirsat and Khanolkar 1962) described the connective tissue fibres in aging and diseased skin and said that the changes induced by these two factors were apparent in the response of the fibres to the classical differential stains for connective tissue. He classified altered fibres on this basis as (a) Elacin - basophilic elastin; (b) Collacin - degenerating collagen retaining its characteristic form but staining like elastin and (c) Collastin - a combination of collagen and elastin staining in one granule.

Gillman, Penn, Bronks and Roux (1954, 1955) in investigations on the extracellular degeneration of connective tissue, using vascular and dermal connective tissue confirmed Unna's results. According to these authors it was possible to distinguish normal collagen and elastin from abnormal altered collagen by their reaction to the differential connective tissue stains in general laboratory use. Morphologically the degenerated collagen appeared coarse, fragmented, thickly beaded and granular. Gillman et al., (1955) were of the opinion that these fibres arise primarily from alteration

in the preformed collagen.

Klemperer (1950) included rheumatism among the so called collagen diseases on the basis of the localisation of the pathological changes occurring in connective tissue. He further stated (Klemperer 1954) that both physical and chemical changes occurred in the collagen component of connective tissue during the fibrinoid degeneration associated with rheumatism. Strukov and Orlovskaya (1957) were of a similar opinion that collagen fibres played a direct role in these fibrinoid changes. (They visualised collagen as being biphasic, consisting of procollagen, with typical "collagen" X-ray diffraction patterns and amino acid composition, surrounding an embryonic type termed collastromine, having no typical "collagen" X-ray diffraction pattern).

Fibrinoid has been defined as "an intensely acidophilic dense refractile and homogenous substance with staining characteristics similar to those of fibrin" (Movat 1960). To the histologist the most significant phenomenon common to the connective tissue diseases is the fibrinoid degeneration of the collagenous tissue. However, fibrinoid nodules are by no means a unique characteristic of the so called collagen diseases and apart from microscopically visible collagen fibres the protein of these nodules contains very little hydroxyproline (Bien and Ziff 1951). Consden, Glynn and Stanier (1953) have reported similar findings and from the amino acid composition of the material they have in fact

concluded that the amount of fibrinoid or fibrinogen derivatives in these nodules must be small. However, the fact that fibrinogen, or material derived from it, was present in these nodules has been demonstrated qualitatively by Gitlin, Craig and Janeway (1957) using fluorescent antibody techniques.

Kellgren, Ball, Astbury, Reed and Bolghton (1951) have suggested that the collagen is replaced by the deposition of fibrinoid and this was evidenced by a decrease in hydroxyproline and an increase in tyrosine during the course of the disease, (see also Kellgren 1955). These observations were suggested by Frey (1963) to explain the different results of many authors in the study of these diseases.

The opposite view was held by some authors (Altschuler and Angevine 1949; Rubens-Duval and Villiaumey 1952) that the ground substance of the connective tissue played the major role in the development of fibrinoid changes.

Sirsat and Khanolkar (1962) have listed a number of authors who have not found any change in the macroperiod of the collagen fibril from many pathological tissues during electron microscopic examination. The disorders cited included Lupus erythematosus, Rheumatoid arthritis and Scleroderma. (Gale 1951; Seville 1952 Reed, Seville and Tattersall 1953; Popper, Schaffner, Huttorer and Darka 1960; Sirsat and Khanolkar 1957, 1962). Kellgren (1955) has suggested a return of the collagen to a more embryoni

type in the connective tissue diseases after investigations using X-ray crystallography suggesting an involvement of this protein.

Evidence for abnormal polysaccharide metabolism in the systemic connective tissue diseases is scant. DiFerrante (1957) has demonstrated an elevated excretion of mucopolysaccarides in the urine in patients with rheumatoid arthritis and depolymerisation of the hyaluronic acid of joint fluid has also been suggested in this condition, (Ragan and Meyer 1949). However, it has been shown that changes in the physical properties of hyaluronic acid occur in many unrelated types of joint inflammation and no specific pathogenic role could be assigned to depolymerisation (Bollet 1956). Although it is interesting to note that hyaluronidase activity has been demonstrated recently in synovia (Bollet 1963).

It appears therefore, that the component of connective tissue whose primary involvement is associated with rheumatoid arthritis and the connective tissue diseases is still debatable. Klemperer (1962) was still of the opinion that collagen was involved primarily.

The Effect of a Disease State on the Shrinkage Temperature of Collagen from Human Tendon.

Although there is evidence suggesting that little or no change occurs in the collagen of tissues affected by the connective tissue diseases the weight of evidence in the other direction would seem to indicate some definite involvement of collagen in these processes.

Studies on the collagen in rheumatic conditions by Rigby and Spikes (1960) have suggested a loss of stability and consequent alteration of collagen under these conditions. The authors have suggested that consequent to a decrease in the hydroxyproline content of affected tissue in rheumatoid arthritis there was a loss of stability which resulted in a gradual denaturation of the collagen at body temperature due to the fact that denaturation and shrinkage of collagen is a time dependent process (Weir 1949). These deductions were based on the linear relationship between shrinkage temperature and hydroxyproline content of collagen, (Rigby and Spikes 1960; Gustavson 1955c). Rigby and Spikes (1960) and Rigby, Hiral, Spikes and Eyring (1959) further suggested that if the structures happen to be under tension then the denaturation process may be accelerated. They have applied this to the phenomenon of heart disease and suggested that the stress mechanism could be a factor in maintaining the predominance of rheumatic complaints in the joints. It was also suggested that if the postulated increase in hydroxyproline during aging

did not take place in any particular person that person might be more liable to become a rheumatic in later life. These observations have been severely criticised by Klein and Curtiss (1962) on the basis that denaturation of collagen as a function of temperature is too broad a phenomenon to explain the specific lesions observed in the rheumatic diseases, and that articular cartilage collagen appeared to be much more stable than tendon collagen. However, Klein (1962) suggests that denaturation may not occur in vivo, although this process coupled with non-specific protease activity, might explain the presence of hydroxyproline in urine found by some authors (Prockop and Sjoerdsma 1961; Lindstedt and Prockop 1961; Ziff, Kibrich, Dresner and Gribetz 1956) and the increase in hydroxyproline excretion after severe burns (Klein, Curtiss and Davis 1962).

Further studies by Rigby (1961 and 1962a) showed that if the collagen is under mechanical stress there is a structural breakdown about 23 centigrade degrees below the shrinkage temperature which is also correlated with the hydroxyproline content. Thus there is the possibility of some form of physical alteration of collagen occurring in the tissue due to a prior chemical deficiency occurring. These processes could only occur in a protein having such a low turnover rate and, as Rigby (1962b) suggests, pH changes in tissue could also have an additive or individual effect on the physical alteration of this protein.

Brown, et al., (1958) have observed a decrease in the shrinkage temperature of the different regions of the rheumatoid nodule and shrinkage temperatures similar to normal in the fascia from patients with Rheumatoid arthritis, Rheumatic fever, Dermatomyositis and Polyarteritis nodosa. The variation of shrinkage temperature with age was used as a control in these experiments. Stringer and Highton (1960) also found abnormal shrinkage temperatures in many specimens of abnormal skin including biopsy specimens from a patient with rheumatoid arthritis. However, in these studies no apparent variation in shrinkage temperature with age was taken into account.

It would therefore appear that, in general, a decrease in the shrinkage temperature indicating a decrease in stabilit of the collagen might be present in specimens of collagen from the tissues of rheumatic patients, in particular those with rheumatoid arthritis. These changes may also be associated with the joint tissue, but it is interesting to speculate as t whether such changes would be reflected in the tendons associated with joints.

Studies on Subunit Patterns of Abnormal Collagen

Some interesting results regarding the effect of age and disease states on the α and β components of soluble collagen of human skin have been published by Bakerman (1964, 1965). According to these results he suggested that there was no change in the $\alpha:\beta$ ratio of the collagen with increasi age although extractability was diminished indicating no alteration in intra-molecular cross linking but an increase in the formation of inter-molecular bonds. However, with patients having a variety of diseases, including the "collagen" diseases, it was found that although extractabilit was diminished below that of the normals at any particular ag the ultra centrifuge pattern of the soluble collagens showed a deficiency in the amount of the β component in collagen from the diseased patients and a slightly enhanced amount of α component. He states that the significance of this was difficult to explain, but it might have been due to one of two factors; (1) that strong inter-molecular bonds have formed in collagen in tissue from diseased patients and that these bonds cannot be disrupted by extraction with citrate buffer or, more likely, (2) that inter-molecular bonds have been disrupted in the tissue and that the soluble fraction which is usually extractable is lost from the dermis through resorption, the α component representing the newly synthesise collagen secreted by mature fibroblasts. There is thus a

loss in total citrate extractable collagen, the synthesis not compensating for the losses of citrate soluble collagen. It might be significant that many of the diseased patients had received steroid therapy.

Enzymic studies on Rheumatoid Collagens.

Studies on the enzymic digestion products of rheumatoid and normal collagens have been carried out by Steven (1964b, 1965a). In initial studies on the action of trypsin on gelatins derived from these collagens by autoclaving and purification by the method of Jackson (1957b), the peptide products were separated by electrophoresis followed by chromatography (Ingram 1956, Baglioni, Ingram and Sullivan 1961). The peptide 'maps' so produced contained between 40 and 50 'spots' and the pattern was identical for both types of gelatin except for two differences which consistently appeared. In the 'maps' derived from normal collagen two peptide spots appeared which were entirely absent or only present in trace amounts in the 'maps' from rheumatoid collagen. It would appear from these results that there might be differences in the amino acid composition of the types of collagen although these differences were slight.

The publication of a patent by Nishihara (1963) for the solubilisation of mature insoluble collagen by the use of crude bacterial α-amylase led Steven (1964a) to adapt this method to the solubilisation of human insoluble collagen as a preliminary to further enzymic studies. Steven (1964a) maintained that the enzyme possessed no proteolytic activity when assayed at pH 5.4 in the pH-Stat against gelatin. The technique adopted was to react collagen homogenates with α-amylase at room temperature (about 18 ^{o}C) and pH 5.4 for ninet

hours. Following washing with 5% sodium chloride the suspension was stirred in 0.2M acetic acid for twenty four hours. This extraction was repeated and after centrifugation the supernatants were adjusted to pH 7 or 8 with normal sodium hydroxide at which point collagen precipitated. It was maintained by Steven that no pepetide bonds were broken but that some destruction of covalent cross-linkages resulted leading to extractability in acetic acid. The collagen thus obtained was stable to gelatinisation at 100°-C (pH 7), but after pepsin treatment 60% (w/w) gelatin was formed by this method. The peptides released by pepsin contained a number of tyrosyl residues and it was suggested (Steven 1965a that this indicated a splitting off of the telopeptide by pepsin and subsequent loss of cross-linking of the type proposed by Bensusan and Scanu (1960) involving the tyrosyl residues of collagen and also supported the telopeptide concept of Hodge et al., (1960). Steven (1965a) also claims a difference in the appearance of the fibrils from rheumatoid and normal collagen after this 'Nishihara' treatment when they were viewed in the electron microscope.

The treatment of 'Nishihara' solubilised normal and rheumatoid collagen with pronase followed by gelatinisation o the mixture in boiling water was followed by Steven (1965b). These studies showed that, the rheumatoid samples were subsequently more thermo-labile, in terms of 'gelatin' production, than the normal samples. Assuming pronase to

attack solely the extra-helical telopeptide region of the molecule, this suggested that there was a deficiency of cross-linking in the helical region of the rheumatoid collagen, or that pronase was not attacking the telopeptides of normal collagen leading to relative instability of the rheumatoid collagen. The fact that there was no increase in soluble nitrogen in the case of the normal samples even after 24 hours enzyme treatment suggested the second type of action.

Further work published recently by Steven (1966a) has suggested that there are two types of cross-link in collagen, one type destroyed by hydroxylamine and therefore of the ester type and a second type destroyed by alkali. From results of thermal stability, in terms of gelatin production, after treatment with alkali and hydroxylamine Steven suggested that a hydroxylamine sensitive, alkali stable type of cross-link existed in the normal collagen (after Nishihara treatment) which was absent in the rheumatoid collagen.

In summary the results of both chemical and shrinkage temperature studies on rheumatoid tissue collagen suggest that there is a tendency for the collagen to be less cross-linked than normal tissue collagen. However, the shrinkage temperatur studies which showed a decrease in T_s. were carried out on "collagen" from rheumatoid nodule and the chemical studies indicating a deficiency in one type of cross-link were carried out on knee joint tissue.

Bakerman's results from studies on human skin suggest a different mechanism of modification of the formed collagen.

It would seem therefore that a study of the effects of age and disease on the tendons closely associated with joints might prove useful. Obviously, it might be expected that from an anatomical point of view the collagen from a knee joint, which has among other things a weight bearing function, might have different properties from, say, tendon collagen which has to transmit a pull or the collagen of skin which contributes to the protective function of this organ. However if the effects of rheumatoid arthritis are as widespread in the body as suggested, it would seem reasonable to assume that those tendons closely associated with joints would be liable to the greatest effect, of all body tissues, apart from joint tissue itself.

SHRINKAGE TEMPERATURE.

Whatever the mechanisms, the data presented on the effects of age on the properties of collagen have led to the hypothesis of an intensification of molecular cohesion in the course of aging and of an increase in the number of intra- and inter-molecular cross-links. In studies of collagen these cross-links may be weakened or broken by various means:-

a). By the application of energy in the form of heat.

b). By the use of agents which interfere with the cross-links by direct competition with the valency forces or by the creation of unfavourable steric conditions in the protein chai (For example lyotropic agents which interfere with valency forces).

These methods along with many others have been used to collect data (chemical, morphological and clinical) on the effects of the normal aging process and disease processes on connective tissue and in particular, collagen. However, perhaps the most extensively studied physical property of vertebrate and invertebrate collagens, with respect to its stability, is its ability to shrink when heated to a certain temperature in an aqueous medium. This shrinkage may be observed by simply subjecting a collagen fibre of convenient size to slow heatin At a specific temperature the fibre starts to shrink and over a range of 2-3°C. continues to contract to a final value of about 30% of its original length. The shrinkage temperature (Ts) of a specific collagen is usually quoted as the range of temperature over which the shrinkage occurs, or, in some case

the temperature at which shrinkage commences (the incipient contraction, Verzar, 1964). For most mammalian collagens shrinking occurs between 60°C. and 70°C.

The purpose of these studies has been previously aimed at the elucidation of the properties of leather and the studies themselves have been used most extensively in the industrial tanning of leather, a process which involves the introduction of cross-links. The history of such studies has been outlined by McLaughlin and Theis (1945) and Hobbs (1940). Chater (1929, 1930) was perhaps the pioneer in the application of measurements of shrinkage temperature to rawhide and leather in its various stages of manufacture including the finished product.

From a thermodynamic point of view the fibrous state is artificial and labile and the coiled globular state with a random distribution of the protein chains represents the more probable and stable state of protein configuration. A stretched chain of a high polymer molecule, which is free to take on the most probable form of configuration, will spontaneously revert to a state of the greatest freedom, the folded form of maximum entropy content. Mirsky and Pauling (1936) first emphasised this point and showed that the change from an orientated structure to a random state of configuration of the protein units is a natural consequence of the tendency the structure to increase its entropy.

With reference to collagen, the restraining effect of the fibre bundle weave and the stabilising forces of the inter chain cross-links (intra- and inter-molecular) must first be overcome before the simple chain effect takes place. With an increasing number of cross-links the length of unaffected chain will diminish and the intra-molecular brownian motion will be impeded. In native collagen, therefore, a stage is reached where contraction involves dislocation of bonds and cross links resulting in a loss of importance of entropy changes and an increase in importance of energy effects. In the first phase of contraction the heat of activation dominat the secondary process of folding running its own course spontaneously. By supplying energy in the form of heat (i.e by increasing the kinetic energy of the polypeptide chains) the cross links should be ruptured to an extent sufficient to allow the chains to be freed from their restraint. The fibr configuration will then be able to follow its own course without change in energy.

The primary reaction in shrinkage is in all probability a melting of the hydrated crystallites, the crystalline regio of the collagen molecule. Garrett and Flory (1956), Flory and Garrett (1958) and Witnauer and Fee (1959) have discussed shrinkage as a melting phenomenon and suggested the process to be an outward indication of the collapse of the helical structure of collagen, a macroscopic manifestation of the molecular, collagen → gelatin, transition.

Changes occurring during shrinkage.

Macroscopically the fibre becomes distorted at the temperature of incipient contraction due to the formation of small nodules, these grow to form a gelatinous node. Zones in the neighbourhood of these nuclei are gradually acted on as the nodules grow so that they coalesce and shrinkage takes place.

The shrunken specimen feels gluey and shows a rubber-like elasticity. The tensile strength is greatly lowered. The original resistance of the collagen to trypsin is destroyed (Grassmann 1936). However, marked hydrolytic changes in the form of splitting of peptide bonds do not seem to occur, and the elementary composition, including the nitrogen content, is unchanged (Grassmann 1936). The maximum acid binding capacity is not affected during shrinkage and the water content remains the same (Gustavson 1956). There is loss of the wide-angle X-ray diffraction pattern (Herzog and Gonell 1925, quoted by Harrington and von Hippel 1961) as well as the small angle pattern (Bear 1944; Wright and Wiederhorn 1951).

Gustavson (1956) has suggested that cross-links of the hydrogen bond type are ruptured during shrinkage. This observation is based on the fact that, by titration curve studies, it has been shown that the ionised carboxyl groups become more reactive in the pH range 2.5 - 5. Weir and Carter (1950) claim that only hydrogen bonds are involved in thermal shrinkage.

Shrinkage as a rate process.

Published results of Chater (1938), Harnly and Parker (1945), and Merry (1945) have shown that a chrome-tanned leather, which has a Ts in excess of 100°C. will shrink progressively if the temperature of the bath is maintained at 100°C. for a period of time. These and other similar results (Weir 1948), led Weir (1949) to suggest that there could be no fixed temperature that could be called a shrinkage temperature but that shrinkage was a rate process occurring over a range of temperatures. This suggestion was substantiated by noting the fibre length after various time intervals when it was immersed in an aqueous medium at certain temperatures, the acceleration of the shrinkage could be noted at each increasin temperature.

That collagen will shrink in time on prolonged storage at temperatures below the normally accepted shrinkage temperature has been noted by Grassmann (1936) and Pankhurst (1947). The process has been termed "incipient shrinkage" by Pankhurst Gustavson (1960) has presented results showing that there is a marked occurrence of disorganisation and impaired stability of skin collagen by water at 45°C. i.e. at a temperature some 25° below the normal shrinkage temperature of skin collagen.

Weir (1949) has shown that, except at low temperatures where shrinkage is quite slow, the shrinkage follows approximately an exponential function of the time, after a short

period of induction. The relationship was written:-

$$l = (l_o - l_\infty)e^{-Kt} + l_\infty \qquad 1$$

l. is the length of the fibre at time t.

l_∞. is the completely shrunken length.

l_o. is the initial length.

K. is the rate constant for the shrinkage.

As equation 1 was shown to be in the form of a first order rate equation a graph of log $(l - l_\infty)$ as ordinate and t as abscissa should yield a straight line of slope -K. This was shown to be the case apart from some non-conformity at very low values of t.

It was found that graphs of data at lower temperatures showed negligible linearity and therefore did not yield values of K. Also at higher temperatures adequate linearity was attained but shrinkage occurred so rapidly that it was difficult to obtain sufficient data to permit the accurate evaluation of K. Weir (1949) therefore utilised the time of half shrinkage as a measure of the rate of the reaction. This was designated $t_{\frac{1}{2}}$ and defined as the time, t, at which the length, l, of the fibre is equal to $\frac{(l_o + l_\infty)}{2}$

The temperature dependence of K was given by the equation:

$$\ln K = \frac{\Delta H}{RT} + C \qquad 2$$

where H represents the heat of activation and is within 1 kcal./mole. of being numerically equal to E, the energy of activation.

R is the gas constant.

T is the absolute temperature.

C is a constant.

A similar relationship was shown to hold if $\ln \left(\frac{1}{t^*}\right)$ was substituted for ln K, where t^* was defined as any correspondi time in the course of the reaction. This time was taken to l a measure of the extent of the reaction and the time corresponding to half shrinkage, $t_{\frac{1}{2}}$, was used by Weir. He goes on to state that because general protein denaturation has been foun to follow the law of a first order reaction (see Neurath, Greenstein, Putnam and Erickson 1944), and the data presented indicated that shrinkage also partly followed this law, then it could be assumed that the shrinkage process is a denaturation reaction of first order and that the times of half shrinkage obtained from experimental data represented times of half chemical reaction.

The linearity of graphs obtained by plotting $\log \left(\frac{1}{t_{\frac{1}{2}}}\right)$ a ordinate and $\frac{1}{T}$ as abscissa was suggested by Weir to justify the validity of use of $t_{\frac{1}{2}}$.

According to Weir (1949) the fact that the visible shrinkage process could only be measured over a short temperature range implied a large heat of activation. He suggeste therefore, on the basis of the theory of absolute reaction ra that in order for such a process to occur with measurable velocity at ordinary temperatures a large entropy increase,

must be associated with activation. In other words the free energy of activation defined by the relationship:

$$\Delta F = \Delta H - T \Delta S \qquad 3$$

must be of a much smaller magnitude than ΔH.

The fundamental equation of the theory of absolute reaction rates used to obtain these quantities from experiment data was quoted as :

$$K = \frac{k T}{h} X K' \qquad 4$$

Where K is the reaction velocity.

k is Boltzmann's constant.

h is Planck's constant.

T is the absolute temperature.

K' is the equilibrium constant of the reaction involving formation of an "activated complex".

X is the transmission coefficient. (This factor was assumed to be unity and constant under the conditions of the experiments).

From the relationship :

$$\Delta F = - R T \ln K' \qquad 5$$

the following substitutions were then made.

Substituting for K' in equation 4:

$$K = \frac{k T}{h} e^{- \Delta F/RT} \qquad 6$$

Substituting for ΔF from equation 3

$$K = \frac{k T}{h} e^{- (\Delta H/RT - \Delta S/R)} \qquad 7$$

Relating K and $t_{\frac{1}{2}}$ by the equation:

$$K = \frac{0.6932}{t_{\frac{1}{2}}} \qquad 8$$

(Reference quoted; Gotman and Daniels 1931)

Then substituting in equation 7 and rearranging:

$$\frac{0.6932\ h}{k\ T\ t_{\frac{1}{2}}} = e^{-(\Delta H/RT - \Delta S/R)} \qquad 9$$

By taking logarithms of both sides of equation 9 it was shown that a graph of $\log \frac{0.6932\ h}{k\ T\ t_{\frac{1}{2}}}$ against $\frac{1}{T}$ yielded a straight line having a slope and intercept proportional to ΔH and ΔS respectively.

Thus from the graphs produced Weir was able to calculate a value for the shrinkage temperature of a collagen fibre by computing the temperature at which the time for half shrinkage was one minute. This was a more satisfactory method of expressing the shrinkage temperature than as the range of temperature over which shrinkage occurred which was more generally quoted. Furthermore values of ΔH, ΔS and ΔF_{T_s} could be calculated and related to this shrinkage temperature and the mechanisms involved in shrinkage. Weir (1949) and Weir and Carter (1950) showed that the effects of tanning on collagen fibres were of three types:

a). Decreasing ΔH and ΔS while increasing ΔF_{60}, (inorganic tannages).

b). Increasing ΔH, ΔS and ΔF_{60}, (deamination and postulated organic crosslinking tannages).

c). No simple correlation, (variation of environment

with organic and inorganic solutes and chemical modification other than those above).

Since these determinations by Weir, and Weir and Carter the Arrhenius equation has been applied to the shrinkage process of rat tail tendon by Akeson (1963) and to the effect of heat on swelling of human diaphragm tendon by Kohn and Rollerson (1959). Although the results produced by Kohn and Rollerson were not sufficiently accurate to produ a linear plot Akeson suggested that the technique might have use in the investigations of the effects of age and disease o the collagen of the rat.

EXPERIMENTAL.

1) Preparation of collagen fibres from various sources.

a) Rat tail tendon.

Rats were sacrificed by stunning and bleeding from the neck vessels. The tails were cut off at the base and the skin carefully stripped away. Complete tail tendons were removed by careful dissection ensuring that no crushing or stretching of the tendon occurred. The tendons were washed in distilled water and used for the various experiments immediately.

b) Human tendon.

Preparations were obtained from the following human tissues:

Normal Achilles Tendon and Patellar Tendon from post mortem cases, obtained within two days after death.

Rheumatoid Patellar Tendon from patients with confirmed rheumatoid arthritis, obtained at operation. (Arthrodesis). Rheumatoid wrist, finger and toe tendons were also used.

All tissues were frozen at -20°C. immediately after removal and stored at this temperature until use.

After thawing, the tissues were cut into thin strips in the direction of the fibre length and the majority of the fat cut away. The tissue was then washed in chloroform/methanol (1/1) followed by 50% aqueous ethanol. Thorough washing with several changes of distilled water removed the remainder of these solvents. The tissue was then gently homogenised in 0.2M disodium hydrogen phosphate (Na_2HPO_4) to remove Globulins and

A Table of Rheumatoid Tissue Sources Showing Age of Patient, Duration of Disease, Erythrocyte Sedimentation Rates (E.S.R.) and Results of Sensitised Sheep Cell Tests (S.S.C.T.).

PATIENT	AGE (Yrs.)	DURATION OF DISEASE (Yrs.)	E.S.R. mm/hr.	S.S.C.T.
D.G.	22	10	29	Neg.
A.C.	29	6	-	-
M.O.	33	14	33	Neg.
R.W.	40	-	-	-
E.H.	41	13	30	Pos.
E.K.	41	14	21	Neg.
C.C.	42	20	42	Pos.
C.G.	43	11	101	Neg.
H.H.	43	11	12	Neg.
E.B.	48	7	50	Neg.
M.C.	52	13	67	Neg.
J.S.	55	15	49	Neg.
A.S.	55	5	30	Pos.
M.C.	56	10	11	Pos.
I.S.	57	8	4	Neg.
R.T.	58	10	47	Pos.
A.H.	59	15	20	Neg.
A.R.	59	1	17	Pos.
G.B.	60	6	19	Pos.
A.M.	60	-	-	-
M.M.	62	7	24	-
H.B.	62	15	12	Neg.
C.M.	63	21	35	Neg.
J.B.	64	12	38	Neg.
D.G.	69	25	12	Neg.

other non-collagenous proteins and the suspension centrifuged at high speed to compress the tissue into a hard pellet. This process was repeated until the supernatant was colourless and protein free and the collagen fibres pure white in colour. Finally thorough washing in distilled water was carried out to remove all the remaining phosphate. The collagen fibres were stored in distilled water at 2°C. until use. With reference to shrinkage temperature determinations it was found by control experiments that this treatment did not alter the T_s values of the resulting fibres and also that storage at 2°C. did not affect those values. It was necessary to use this method of pretreatment as it led to a mass of partially separated collagen fibres of fairly uniform thickness, a convenient starting material for subsequent determinations.

c) <u>The preparation of collagen fibres after the "Solubilisation" of Insoluble Collagen fibres with Crude Bacterial α-Amylase.</u> (The "Nishihara technique", Steven 1964a).

The treatment of insoluble collagen using α-amylase to produce a soluble type of collagen was patented by Nishihara (1963) and was adapted by Steven (1964a) for the conversion of human insoluble collagen to a soluble form. It was considered interesting to determine the effects of this treatment on the chemical nature and properties of human insoluble collagen from normal and rheumatoid sources of different ages.

<u>Method</u>

Human tendon was pretreated as described previously to

produce a mass of clean white collagen fibres. Aliquots of these fibres were taken for the α-amylase treatment, washed in distilled water and then suspended in 0.22M NaH_2PO_4 adjusted to pH 5.4 (pH meter). To this suspension 0.3% (w/w) crude bacteria α-amylase (Cambrian Chemicals) was added. The mixture was stirred at room temperature (18°C) for 90 hours. The collagen fibres were then washed in 0.2M Na_2HPO_4 followed by distilled water and finally suspended and homogenised in 0.16M acetic acid. The resultant suspension was stirred for 24 hours and then the residue centrifuged down and re-extracted with acetic acid. The supernatants were combined and the soluble collagen precipitated by addition of sodium chloride and simultaneous gentle stirring of the solution. The fibres so formed were washed in distilled water and dialysed against distilled water until completely salt free.

2. Expression of Results

In the various determinations carried out on collagen from human sources the results have been expressed, where applicable, as a function of the age of the tissue source. In doing this the different ages have been grouped together in the following manner:-

1) Ages 0 to 6 months. (Including foetal ages 8 to 9 months).

2) Ages 7 months to 10 years.

3) Ages 11 to 20 years.

4) Ages 21 to 30 years.

up to

9) Ages 71 to 80 years.

Samples were obtained from patients with rheumatoid arthritis in the age range 22 - 69 years. There are therefore only 5 age groups for results from rheumatoid collagen. In some instances results have been presented for the normal collagens over these 5 age groups only.

Results were obtained from samples of collagen in duplicate, and at least four samples of different ages were included in each age group.

3. Analytical Methods.

The Determination of Ash and Moisture Content of Human Tendon Collagen Samples

a) The Determination of Ash Content.

Freeze dried samples of pretreated normal and rheumatoi tendon tissue from sources of a series of different ages were carefully weighed in a porcelain crucible. The crucible was then heated, first gently and then more strongly over a bunsen flame ensuring that no solids were lost by convection. The crucible and ash were then transferred to a high temperature oven and heated at 400°C. to a constant weight.

b) The Determination of Moisture Content.

About 500mg. of air dried collagen fibres were weighed out accurately in a preweighed stoppered weighing bottle. The unstoppered weighing bottle and sample were then placed in an oven set at 105°C. After 6 hrs. the weighing bottle was stoppere and allowed to cool in a dessicator containing fused calcium chloride. After weighing the unstoppered bottle was returned to the oven and this process repeated until a constant weight was obtained.

Results.

The results of these determinations were expressed as a percentage of the air dried sample. Air dried samples were prepared by freeze drying samples of collagen and then storing these in a dessicator with a water saturated atmosphere at 18°C. until a constant weight was obtained.

The Determination of the Total Nitrogen Content of Human Tendon Collagen.

Freeze dried pretreated tissue samples (about 200 mg.) were hydrolysed in 2 ml. of concentrated, nitrogen free, sulphuric acid for 18 hrs. in standard micro-kjeldahl tubes in the presence of a small quantity of "kjeldahl catalyst" (Copper-Selenium). The resultant hydrolysate was transferred quantitatively to the standard distillation apparatus and steam distilled in the presence of 10 ml. of 40% potassium hydroxide (Analar). The liberated ammonia collected was by passing the distillate into about 10 ml. of distilled water (pH 5.5) containing a small amoun of Tashiri's indicator. The solution was then titrated back to the original indicator colour with N/70 sulphuric acid (5 ml. of acid equivalent to 1 mg. of nitrogen).

These results were expressed as a percentage of the dry protein weight.

Preparation of Samples for Further Chemical Analysis.

a) Acid Hydrolysis for Hydroxyproline Determinations.

About 100 mg. of freeze dried collagen were hydrolysed in a sealed tube (pyrex tube fitted with PTFE washer and screw cap in 20 ml. of 6N. HCl for 18 hrs. at 110°C. After cooling the solution was made up to 100 ml. with distilled water for the determinations.

b) Acid Hydrolysis for Amino Acid Analyses.

About 50 mg. of freeze dried collagen were hydrolysed with 15 ml. of 6N. HCl in a sealed tube of the type already described, for 40 hrs. at 110°C. The hydrolysate was then

transferred to the container of a rotary film evaporator and the acid distilled off under vacuum with repeated addition of distille water until the distillate was acid free. The residue was then made up to a suitable volume for the analyses, usually about 10 ml

c) Resin Hydrolysis.

This method was used in comparative estimations of hexose, hexosamine and hydroxyproline content, and was a modified method of Anastassiadis and Common (1958).

Dowex 50 resin (200-400 mesh) was prepared in the H^+ form by washing repeatedly with 2N. NaOH on a buchner filter, followed by several washings with 2N. HCl. The resin was then washed with distilled water until all the washings were neutral. The resin was then suspended in 0.05N. HCl (2/1 volume of acid to weight of resin).

About 100 mg. of freeze dried collagen was hydrolysed with 10 ml. of the resin suspension in a sealed tube, already described, for 40 hrs. at 110°C. After cooling the tubes were opened, the suspension mixed and the contents transferred quantitatively with 20 ml. of distilled water to a column 10 mm. in diameter. The eluate was collected in a conical flask and constituted "water eluate 1". The flask was then replaced by a 50 ml. volumetric flask and elution carried out with 2N. HCl followed by a further 10 ml. of distilled water. This constitute "acid eluate 1". This volumetric flask was replaced by a second 50 ml. volumetric flask and "water eluate 1" added to the column. A further 20 ml. of distilled water was added to the column and

the eluate collected and made up to the 50 ml. This constituted "water eluate 2". The volumetric flask containing "acid eluate 1" was replaced under the column and a further elution carried out with 20 ml. of 2N. HCl. After the column had drained the volume in the flask was made up to 50 ml. This constituted "aci eluate 2".

The "acid eluate 2" contained the hexosamine and hydroxyproline constituents, the "water eluate 2" contained the hexose constituents.

Determination of Hydroxyproline.

The method of Neuman and Logan (1950), as modified by Leach (Eastoe and Courts 1963).

Reagents.

0.05M Copper sulphate solution. (12.5 g. $CuSO_4.5H_2O$ in 1 litre distilled water.)

2.5N Sodium hydroxide solution. (100 g. A.R. grade NaOH pellets in 1 litre distilled water).

6% (w/v) Hydrogen peroxide. (30% (w/v) A.R. grade hydrogen peroxide diluted with 4 times its volume of distilled water immediately before use).

3N Sulphuric acid. (83 ml. concentrated H_2SO_4 in 1 litre of distilled water).

5% p-Dimethylaminobenzaldehyde. (5 g. of the reagent in 100 ml. redistilled propan-1-ol).

Standard hydroxyproline solution. (50 mg. L-hydroxy-

proline in acid solution consisting of 400 ml. of distilled water plus 20 ml. of concentrated HCl adjusted to 500 ml. with further distilled water. The concentration is thus 100 µg. per ml.).

Procedure.

The hydrolysate was first diluted with water to yield a solution containing approximately 5-10 µg. per ml. of hydroxyproline. Duplicate 1 ml. samples of the test solution, and of standard solutions containing 5, 10, and 15 µg. per ml. were pipetted into test tubes, and a 1 ml. water blank included as well. Into each was then pipetted 1 ml. of 0.05M $CuSO_4$ followed by 1 ml. of 2.5N NaOH. The contents of each tube were then mixed by gentle shaking and the tubes placed in a water bath at 40°C. After five minutes at this temperature 1 ml. of 6% hydrogen peroxide was added to each tube and the contents immediately mixed by vigorous shaking. The tubes were then returned to the water bath and left for a further 10 min. After this time the tubes were cooled rapidly in iced water. 4 ml. of 3N H_2SO_4 was then added to each tube followed by 2 ml. of 5% p-dimenthylaminobenzaldehyde and the contents thoroughly mixed. The tubes were then loosely sealed and placed in a second water bath at 70°C. for 16 min. After cooling to room temperature (18°C.) the optical densities were read at 555 mµ. in a spectrophotometer.

Comments.

After addition of hydrogen peroxide it was important

that there was no residual solution on the sides of the tubes otherwise incomplete destruction of the peroxide ensued leading to erroneous results.

Determination of Hexose

The Method of Yemm and Willis (1954). Estimation of the colour produced on reaction of anthrone with hexoses in acid solution.

Reagents

Anthrone reagent. (0.2 g. anthrone in 100 ml. sulphuric acid. The sulphuric acid was made by adding 500 ml. conc. H_2SO_4 to 200 ml. of distilled water). Fresh reagent was made up each day and used within 12 hours.

Standard glucose solution. (100 µg/ml. in distilled water).

Procedure.

For each test 5 ml. of anthrone reagent was pipetted into each of nine thick walled Pyrex boiling tubes and these chilled in ice water. 1 ml. of the solution under test was pipetted into each of two tubes to layer on top of the acid, 1 ml. of distilled water in a third tube as a blank and duplicate 1 ml. samples of each of the standard glucose solutions, 10 µg/ml. 30 µg/ml. and 50 µg/ml. were also added to the remaining tubes as indicated. The tubes were cooled for a further 5 minutes and then thoroughly mixed while still being kept cool. Finally the tubes were loosely fitted with corks and heated for 15 minutes in a boiling water bath. After cooling in tap water the optical density was read on a spectrophotometer (SP 500, or 600) at 620 mµ

Comments.

This method gives good consistent results. However, great care should be exercised in the mixing of anthrone reagent and test solution. Wide (2.5 cm.) boiling tubes should always be used.

Determination of Hexosamine.

The Method of Cessi and Piliego (1960). The method was developed primarily to afford a suitable method of hexosamine determination which compensated for the interference of amino acids which are necessarily present in any tissue hydrolysate. The method is based on the separation of volatile chromogens of the amino sugars from the non-volatile chromogens of amino acids. The volatile chromogens are distilled over and form a coloured complex with Ehrlich's reagent.

Reagents.

Acetyl acetone; (Redistilled b.p. 138-140°C. stored at 2°C).

p-Dimethylaminobenzaldehyde reagent; (80 mg. dissolved in 100 ml. of absolute ethanol acidified with 3.5 ml. of conc. HCl). This solution was stable for 3-4 days at 2°C.

Buffer, pH 9.8; (0.5N sodium carbonate - sodium bicarbonate containing 0.1M sodium chloride).

Acetyl acetone reagent; (1 ml. acetyl acetone in 100 ml. buffer, pH checked). This reagent was stable for one day at 2°C.

Standard glucosamine solution containing 100 µg of fre
sugar per ml. of distilled water.

Distillation was carried out in an all glass apparatus
(A 100 ml. spherical flask and a 20 cm. water condenser). The
apparatus was heated directly over a gas flame and distillation
carried out at the rate of about 1 ml/min.

Procedure

Eleven quickfit stoppered test tubes were used.
Duplicate 2 ml. samples of standard solutions (containing 25 µg,
50 µg, 75 µg and 100 µg per ml. of glucosamine) and the test
solutions with one blank of distilled water were pipetted into
separate tubes. 5.5 ml. of acetyl acetone reagent were added
to each tube and the tubes well shaken. The stoppered tubes were
then heated on a boiling water bath for 20 minutes. After
cooling, the reaction mixture plus three 2 ml. washings of
distilled water were transferred to the distillation flask. 2
ml. distillates were distilled over (in about 2 minutes) into a
10 ml. volumetric flask containing 8 ml. of p-dimethylamino-
benzaldehyde reagent. These flasks were then stoppered, shaken
and the colour read after 30 minutes in a spectrophotometer at
545 mµ.

Amino Acid Analyses.

Method.

A Technicon automatic amino-acid analyser was used
for these determinations.

Results.

Representative results are shown in Table 6. Correction for loss of threonine and serine during hydrolysis was carried out using the values suggested by Serafini-Fracassini Tristram and Agnew (1965).

4) Analytical Results

TABLE 4

The Proportion of Ash, Moisture, Hydroxyproline, Hexose, Hexosamine and Total Nitrogen in Normal and Rheumatoid Human Tendon Collagens from Tissue Sources of Different Ages.

	Ash		Moisture		Hypro		Hexose		Hexo-samine		T.N.	
	N	R	N	R	N	R	N	R	N	R	N	R
0-6 mths.	Nil	-	19.0%	-	7.5%	-	0.5%	-	Nil	-	18.0%	-
7 mths.-10 yrs.	Nil	-	20.0%	-	8.0%	-	0.7%	-	Nil	-	18.2%	-
11-20 yrs.	Nil	-	15.0%	-	8.0%	-	0.6%	-	Nil	-	17.9%	-
21-30 yrs.	Nil	Nil	17.0%	12.0%	7.9%	7.5%	0.65%	0.6%	Nil	Nil	17.7%	17.9%
31-40 yrs.	Nil	Tr.	15.0%	14.0%	9.0%	8.5%	0.7%	0.75%	Nil	Nil	17.9%	17.8%
41-50 yrs.	Tr.	Nil	11.0%	10.5%	9.5%	9.0%	0.75%	0.7%	Nil	Nil	18.1%	18.0%
51-60 yrs.	Nil	Nil	12.0%	10.0%	9.5%	7.5%	0.65%	0.65%	Nil	Nil	18.0%	18.2%
61-70 yrs.	Nil	Nil	10.0%	8.5%	11.0%	7.0%	0.7%	0.7%	Nil	Nil	18.1%	17.9%
71-80 yrs.	Tr.	-	11.0%	-	12.0%	-	0.7%	-	Nil	-	17.8%	-

Notes.

N = Normal Collagen. R = Rheumatoid Collagen. Tr. = Trace.

Moisture is expressed as a percentage of the air dry protein weight.

Hydroxyproline, Hexose, Hexosamine and Total Nitrogen were expressed as percentages of the dry protein weight.

TABLE 5

The Proportion of Ash, Moisture, Hydroxyproline, Hexose, Hexosamine and Total Nitrogen in Normal and Rheumatoid Human Tendon Collagens from Tissue Sources of Different Ages, After Treatment with -Amylase

	Ash		Moisture		Hypro		Hexose		Hexosamine		T.N.	
	N	R	N	R	N	R	N	R	N	R	N	R
0-6 mths.	Nil	-	20.0%	-	9.0%	-	0.1%	-	Nil	-	17.7%	-
7 mths. - 10 yrs.	Nil	-	18.0%	-	8.5%	-	0.1%	-	Nil	-	18.1%	-
11-20 yrs.	Nil	-	19.5%	-	9.5%	-	0.1%	-	Nil	-	17.9%	-
21-30 yrs.	Nil	Tr.	18.0%	14.5%	10.0%	7.5%	0.05%	0.2%	Nil	Nil	18.0%	18.0%
31-40 yrs.	Nil	Nil	16.0%	15.0%	10.5%	9.0%	0.2%	0.2%	Nil	Nil	18.2%	18.0%
41-50 yrs.	Nil	Nil	17.0%	12.0%	10.0%	10.0%	0.15%	0.15%	Nil	Nil	18.2%	18.2%
51-60 yrs.	Tr.	Nil	16.5%	12.0%	12.0%	8.0%	0.3%	0.1%	Nil	Tr.	17.8%	18.3%
61-70 yrs.	Tr.	Nil	14.5%	10.0%	11.5%	9.0%	0.35%	0.15%	Nil	Nil	18.0%	17.9%
71-80 yrs.	Nil	-	14.0%	-	13.0%	-	0.35%	-	Nil	-	17.8%	-

Notes.

N = Normal Collagen. R = Rheumatoid Collagen. Tr. = Trace.

Moisture is expressed as a percentage of the air dry protein weight.

Hydroxyproline, Hexose, Hexosamine and Total Nitrogen were expressed as percentages of the dry protein weight.

TABLE 6

The Amino Acid Composition of some Normal and Rheumatoid Collagen

Values are given as g. amino acid per 100g. dry protein

	Normal		Rheumatoid	
	11-20	61-70	41-50	51-60
Glycine	27.7	25.4	28.5	28.2
Alanine	10.4	12.3	11.2	10.9
Valine	3.0	3.2	3.5	3.6
Leucine	3.5	3.5	3.6	3.9
Isoleucine	1.5	1.9	1.7	1.7
Phenylalanine	2.2	2.4	2.4	2.3
Tyrosine	0.7	0.3	0.6	0.3
Serine	4.3	4.1	2.0	1.5
Methionine	0.7	-	Tr.	Tr.
Threonine	1.8	2.0	1.5	1.5
Proline	19.5	19.4	21.1	20.8
Hydroxyproline	8.2	10.7	8.5	8.0
Aspartic acid	5.6	5.3	6.1	6.5
Glutamic acid	11.1	10.4	11.1	11.7
Arginine	8.7	9.1	8.6	8.4
Histidine	1.0	0.9	1.0	0.9
Lysine	3.7	3.6	3.5	3.8
Hydroxylysine	1.4	-	1.7	1.5

Notes: Figures quoted are averages of values obtained for several tissues within the age range quoted. Values above 1g/100g. showed a variation of $\pm$ 0.5g./100g. Values for Tyrosine and Methionine were unreliable.

5) Solubility Studies

The Solubility of Normal and Rheumatoid Human Tendon Collagen in Neutral Salt Solution (1M NaCl), Citrate buffer (pH 3.8) and Acetic acid (0.16M), and the Effect of Age.

Method.

Samples of normal and rheumatoid collagen of different ages (about 2g. dry weight) were suspended in distilled water at room temperature (18°C) for 24 hrs.

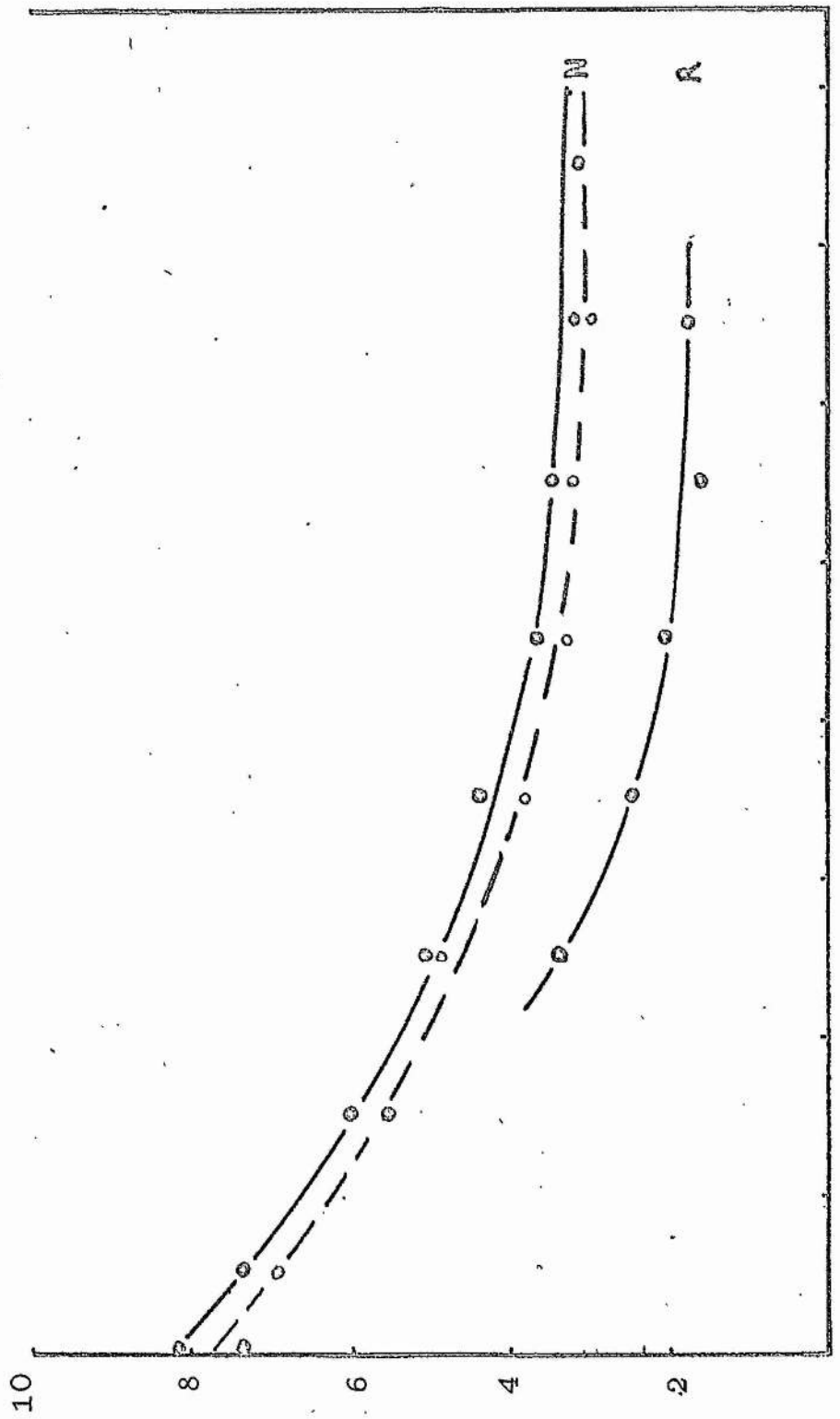
Aliquots of these samples were homogenised in 50 ml. of neutral salt, citrate buffer or acetic acid for periods of 10 minutes. The resultant homogenates were then shaken on a microid flask shaker for 24 hrs. at room temperature. The suspension was then centrifuged at 35,000 x g for 1 hr. and the whole procedure repeated once more on the residue. The collagen content of the supernatant was then determined by estimating the "Total Nitrogen" and using the figure of 18% for the content of nitrogen in collagen. The solubility was expressed as mg. of collagen solubilised per g. of dry collagen.

Results.

The results were expressed graphically (Graph 1). It was found that the quantity of collagen solubilised by neutral salt was so low as to be almost insignificant (less than 0.5 mg./g dry collagen) and as there was no obvious correlation between solubility in this solvent and age the results were not included in the graph for the sake of clarity.

GRAPH 1

The solubility of tendon collagen in acetic acid and citrate buffer.



Age in years

mg. of collagen dissolved/g. dry collagen

N = Normal collagen R = Rheumatoid collagen

—— Acetic acid (0.16M) ---- Citrate buffer (pH 3.8)

In the case of rheumatoid collagen no difference in solubility was apparent in acetic acid and citrate buffer.

Treatment of Normal and Rheumatoid Collagen of Different Ages with Hyaluronidase, Trypsin or Papain.

Method.

Aliquots of collagen were suspended in 50 ml. of suitable buffer (see below) and enzyme added in an enzyme; substrate ratio of 1:100. The flasks were shaken for 48 hrs. at room temperature The suspensions were then centrifuged at 35,000 x g for 1 hr. Th residue was washed once with appropriate buffer and spun down once more. The two combined supernatants for each sample were tested for hydroxyproline by the method of Neuman and Logan (1950) after evaporating to dryness, desalting and mild acid hydrolysis to convert any peptide bound hydroxyproline to free hydroxyproline.

Treatment with hyaluronidase (Ovine testis B.D.H. Optimum pH 5.5 - 6.2) was carried out in 0.1M phosphate buffer pH 5.8.

Treatment with trypsin (Pancreatic DIFCO. control 47538. Optimum pH 7.0-8.0) was carried out in 0.1M phosphate buffer pH 7.5.

Treatment with papain (B.D.H. Optimum pH, wide range) was carried out in 0.1M phosphate buffer pH 6.

Results.

After hyaluronidase treatment there was apparently no hydroxyproline released into the supernatant in any of the samples rheumatoid or normal. However trypsin and papain both released small quantities of this amino acid from collagen.

The results could not be expressed graphically as there appeared to be only slight correlation with age in the case of

trypsin treatment. These are shown as quantity of hydroxyproline released per g. dry collagen from tissue sources up to age 50 yrs. and above age 50 yrs.

Trypsin: Age groups below 50 yrs. 0.12 mg. hydroxyproline/g. dry collagen (for normal and rheumatoid)

Trypsin: Age groups above 50 yrs. 0.09 mg. hydroxyproline/g. dry collagen (for normal and rheumatoid)

Papain: All age groups 0.21 mg. hydroxyproline/g. dry collagen (for normal and rheumatoid)

<u>The Solubility of Normal and Rheumatoid Collagen in Neutral Salt Solution (1M NaCl), Citrate Buffer (pH 3.8) and Acetic Acid (0.16M) after Treatment with Hyaluronidase, Trypsin or Papain, and the Effects of Age.</u>

Method.

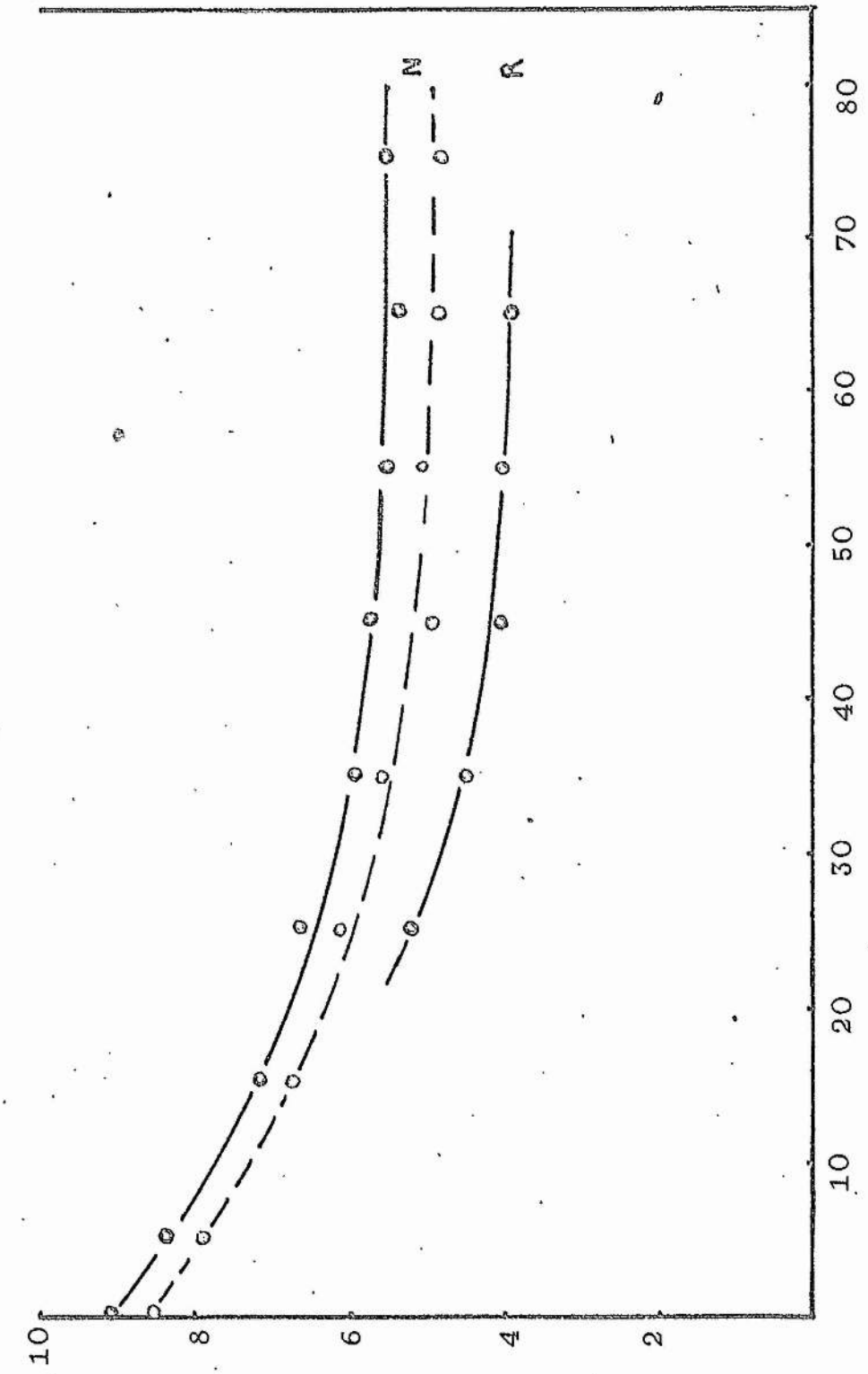
Aliquots of the residues after enzyme treatment were washed with distilled water to remove traces of the enzymes and then dialysed against distilled water to remove the phosphate. The samples were then homogenised in 50 ml. of the appropriate solvent for periods of 10 minutes, and the resultant homogenates shaken for 24 hrs. at room temperature. After centrifuging at 35000 xg for 1 hr. the procedure was repeated on the residue. The collagen content of the combined supernatants for each sample was determined by estimation of the "Total Nitrogen" (assuming 18% T.N. for collagen). The solubility was expressed as mg. of collagen solubilised per g. of dry collagen before enzyme treatment.

Results.

The results were expressed graphically (Graphs 2, 3 and 4 Here again the neutral salt soluble fraction of enzyme treated collagen proved to be very low, although possibly a little more than before enzyme treatment, it amounted to less than 1.5 mg./g. of dry collagen. Correlation with age was also not apparent and therefore these values were not included in the graphs.

GRAPH 2

The solubility of tendon collagen in acetic acid and citrate buffer after hyaluronidase treatment.



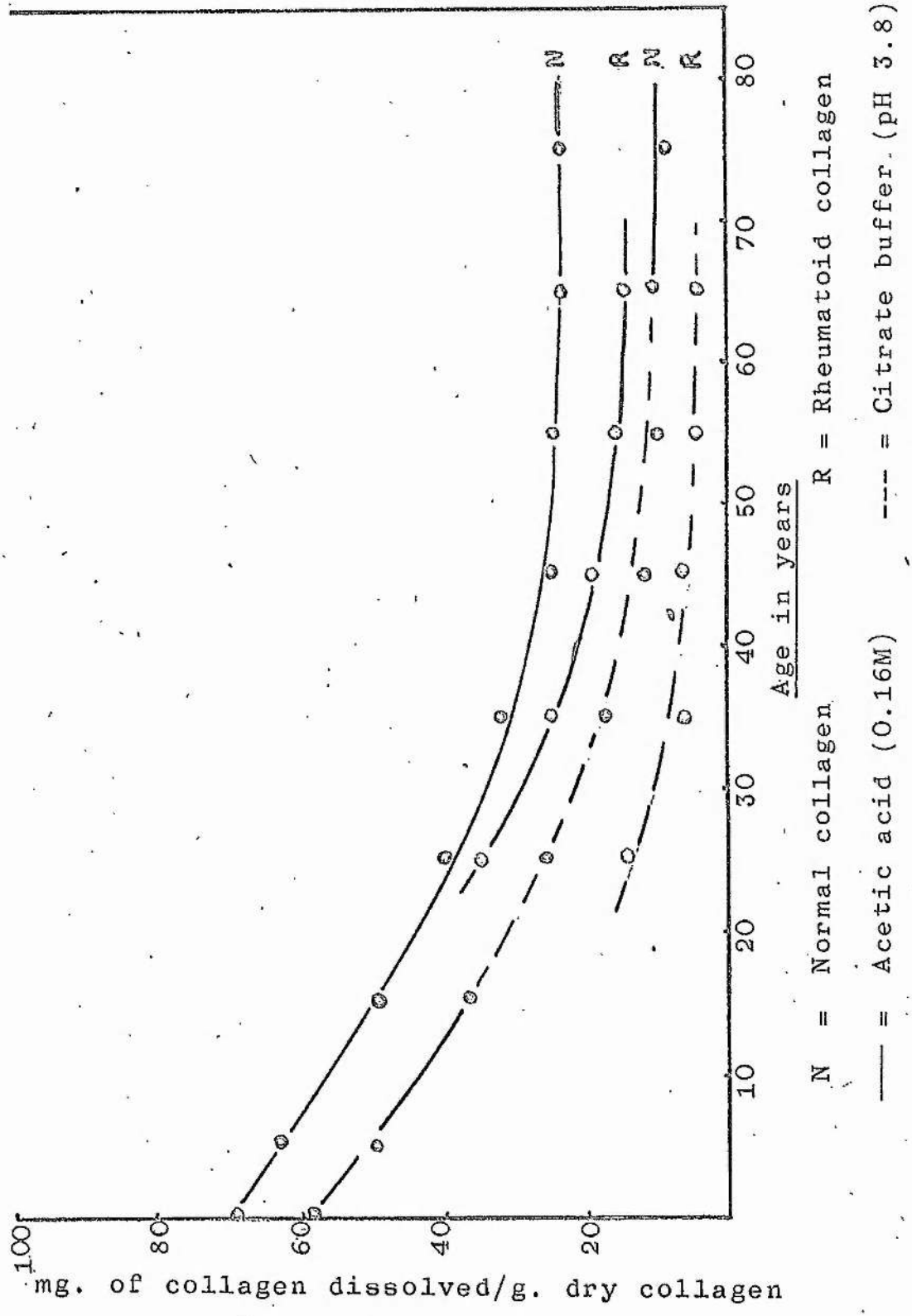
N = Normal collagen R = Rheumatoid collagen

—— = Acetic acid (0.16M) ---- = Citrate buffer (pH 3.8)

In the case of rheumatoid collagen no difference in solubility was apparent in acetic acid and citrate buffer.

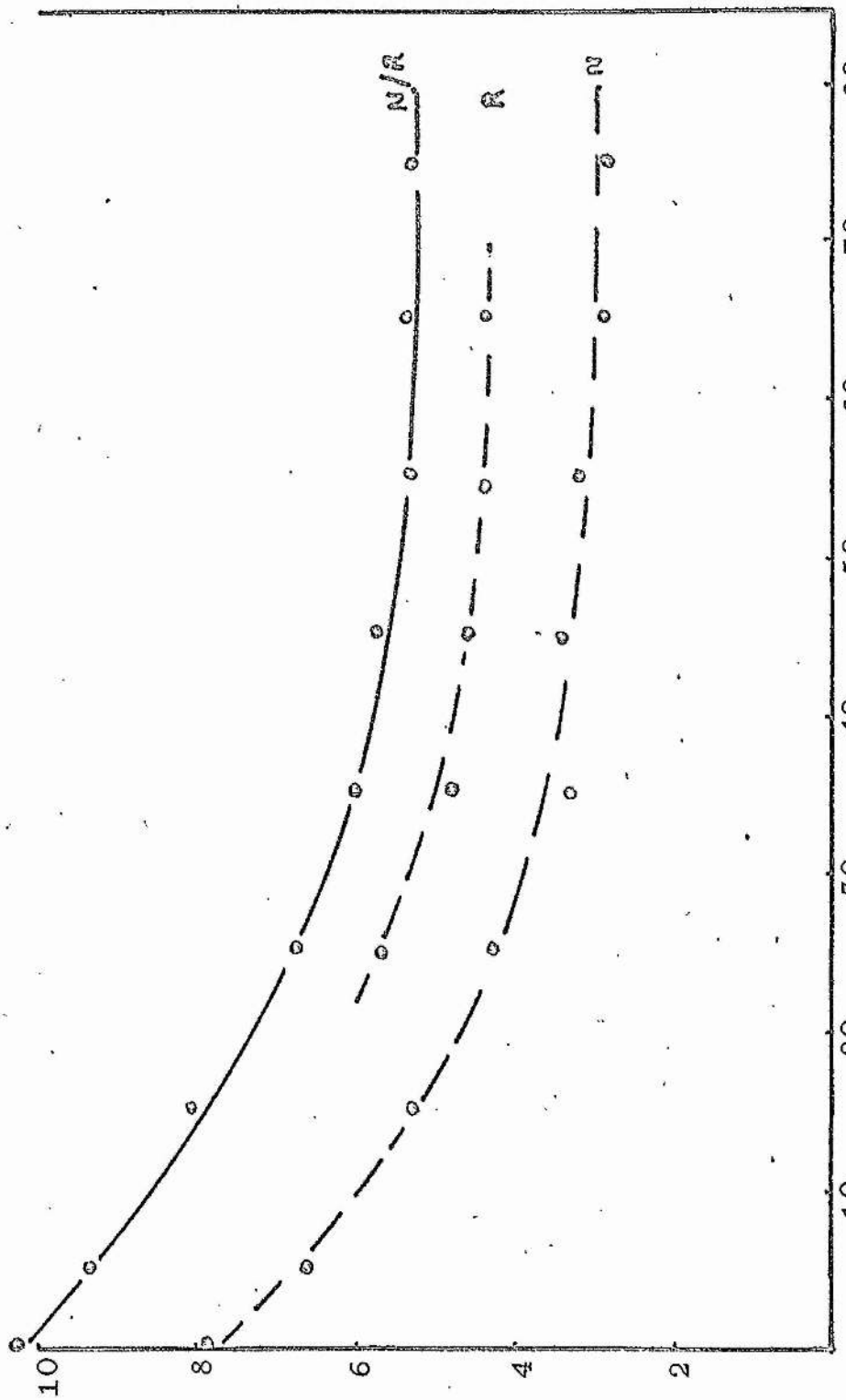
GRAPH 3

The solubility of tendon collagen in acetic acid and citrate buffer after trypsin treatment.



GRAPH 4

The solubility of tendon collagen in acetic acid and citrate buffer after papain treatment.



mg. of collagen dissolved/g. dry collagen

N = Normal collagen R = Rheumatoid collagen.

—— = Acetic acid (0.16M) ---- = Citrate buffer (pH 3.8)

No difference in solubility in acetic acid was apparent with normal and rheumatoid collagen.

6) Shrinkage Temperature Studies

Preliminary studies on the Influence of Age on the Shrinkage Temperature (Ts) of Rat Tail Tendon Collagen.

Tail tendons were obtained from Albino Rats of different ages as already described. The rats were selected according to age and weight as shown below, usually four rats were used of each age.

1 month	weight 64 - 70 g.	average weight 67 g.
2 months	weight 194 - 250 g.	average weight 219 g.
6 months	weight 250 - 313 g.	average weight 281 g.
9 months	Weight 346 - 380 g.	average weight 360 g.
12 months	weight 480 - 520 g.	average weight 494 g.
18 months	weight 485 - 570 g.	average weight 532 g.

The shrinkage temperature determination was based on the technique used by Brown, Consden and Glynn (1958). The fibres were cut to a length of 2 cms. and threaded into Westergren Sedimentation Rate tubes (conveniently calibrated in millimetres and centimetres). The tubes were then suspended in a glass sided water bath, filled with glass distilled water and fitted with a Sunvic Thermostat control and 500 watt immersion heater. Temperature control for constant values in the range 55 - 70°C was within 0.1 of a Centigrade degree.

After allowing sufficient time for the fibres to adjust to the temperature of the bath (18-20°C) the temperature of the bath was raised at the rate of about 2°C per minute and the temperature noted at the start of contraction (incipient

contraction, Verzar 1964) and the finish of contraction (maximum contraction, Verzar 1964).

Having determined the range of temperature over which the fibres contracted it was found that there was a general increase of the temperature of both incipient and maximum contraction with age, although some overlap occurred, see table of results below.

Age in months	Range of shrinkage
1	58.5 - 61.5°C
2	59.0 - 62.5°C
6	60.0 - 63.0°C
9	60.5 - 63.5°C
12	61.0 - 64.0°C
18	61.5 - 64.0°C

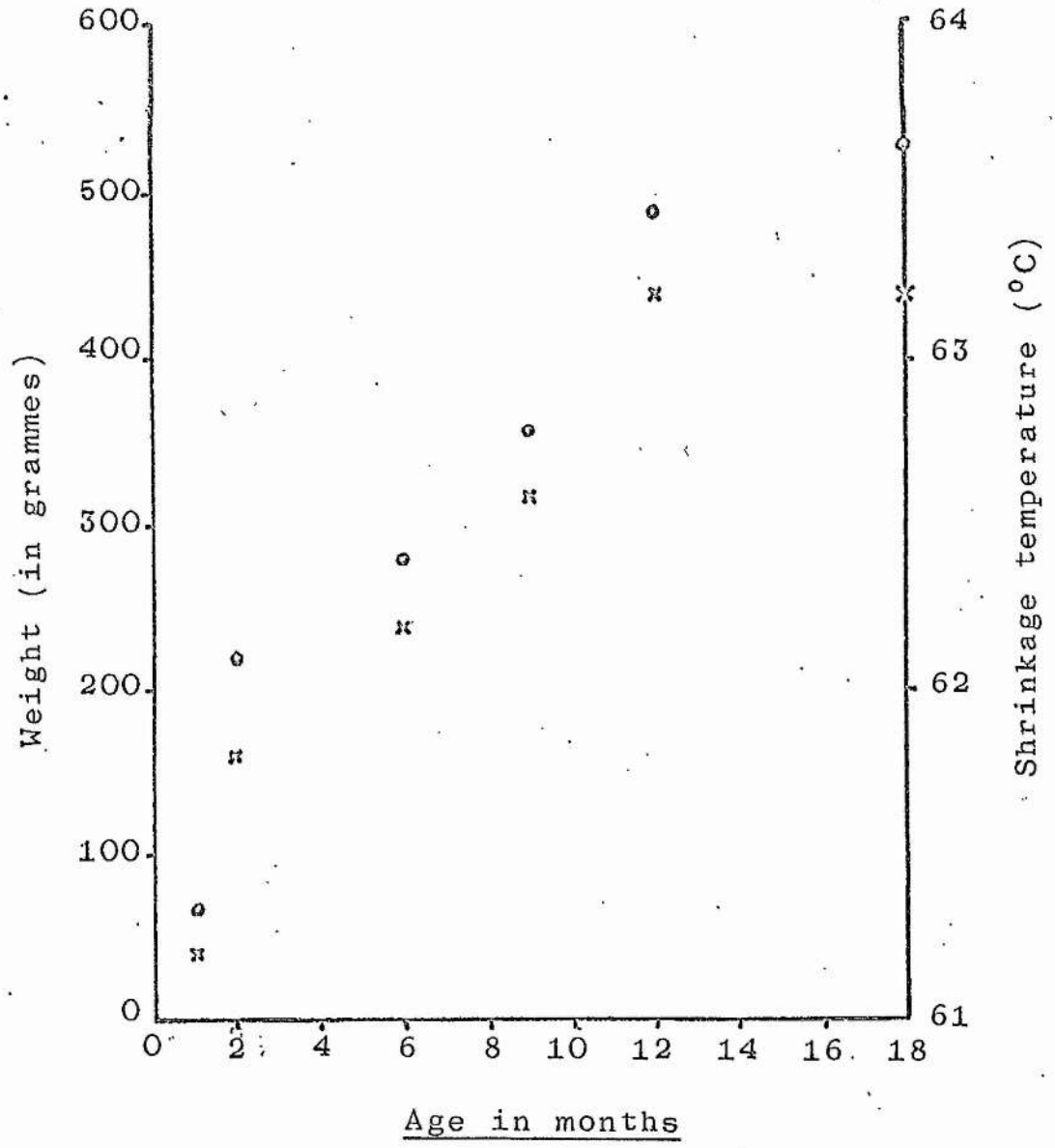
Comparison of results such as those above is difficult and it was therefore considered possible that the temperature at which the time for half shrinkage is 60 seconds (Weir 1949) would produce more comparable values for T_s.

The determination of this value for half shrinkage was carried out on the group of samples from the one month old rats in the following manner. The bath temperature was set accurately at the lower end of the shrinkage temperature range (59°C) and three Westergren tubes containing fibres quickly suspended in the bath. The initial length of each fibre was noted and the length taken at time intervals until shrinkage had ceased. The temperature of the bath was raised to 60°C and a further series

of determinations on a fresh set of fibres carried out. This process was carried out for at least one more temperature within the shrinkage temperature range. The time for shrinkage to half length $\frac{\text{original length + contracted length}}{2}$ was computed for each temperature of the bath. A plot of $\log \frac{1}{t_{\frac{1}{2}}}$ against $\frac{1}{T}$ (where $t_{\frac{1}{2}}$ is the time for shrinkage to half length and T is the absolute temperature) was used to obtain the temperature at which the time for half shrinkage ($t_{\frac{1}{2}}$) was 60 seconds. This temperature is termed the shrinkage temperature in all subsequent results. Averages were taken for all sets of determinations. The shrinkage temperature was determined in this way for fibres from each age of animal. The final value for the shrinkage temperature for each age was plotted against the average weight for the particular age, (Graph 5).

GRAPH 5

A graph showing the variation of rat weight and shrinkage temperature of rat tail tendon collagen with age.



The Effect of Age and Disease on the Shrinkage Temperature of Human Tendon Collagen

Collagen fibres which had been obtained from normal and rheumatoid tissue of different ages as previously described were used in these studies.

Method.

Single fibres were gently teased out of the pretreated tissue samples and soaked for about 1 hour in distilled water. The fibres were then cut to a length of 2 cm. and threaded into Westergren Sedimentation Rate tubes. Shrinkage temperature determinations were carried out immediately by the method already described for rat tail tendon fibres. The range of temperature over which shrinkage occurred was determined for several fibres from each age group and then the $t_{\frac{1}{2}}$ values were determined at temperatures within these ranges.

Results.

The Temperature Range of Shrinkage for Human Normal Tendon.

Age	Shrinkage Range	Age	Shrinkage Rang
0 - 6 mths.	59-60°C	41-50 yrs.	63-65°C
7 mths.-10 yrs.	60.5-62°C	51-60 yrs.	64-66°C
11 - 20 yrs.	61-63°C	61-70 yrs.	64.5-66.5°C
21 - 30 yrs.	62.5-64°C	71-80 yrs.	65-66.5°C
31 - 40 yrs.	63-64.5°C		

Plots of $\log \frac{1}{t_{\frac{1}{2}}}$ against $\frac{1}{T}$ are shown in Graphs 6 and 7.

Plots of the variation of shrinkage temperature with age for normal and rheumatoid tissue sources are also presented. (Graphs 8 and 9).

Here again averages were taken for all sets of determinations and furthermore the values for the shrinkage temperature were adjusted to the nearest 0.25 of a degree. Ages were grouped together in the manner mentioned previously.

GRAPHS 6 & 7

Plots obtained for:

$\log \frac{1}{t_{\frac{1}{2}}}$ against $\frac{1}{T}$

for different age groups,

1) 0 - 6 months.

2) 7 months to 10 years.

3) 11 - 20 years.

4) 21 - 50 years.

etc.

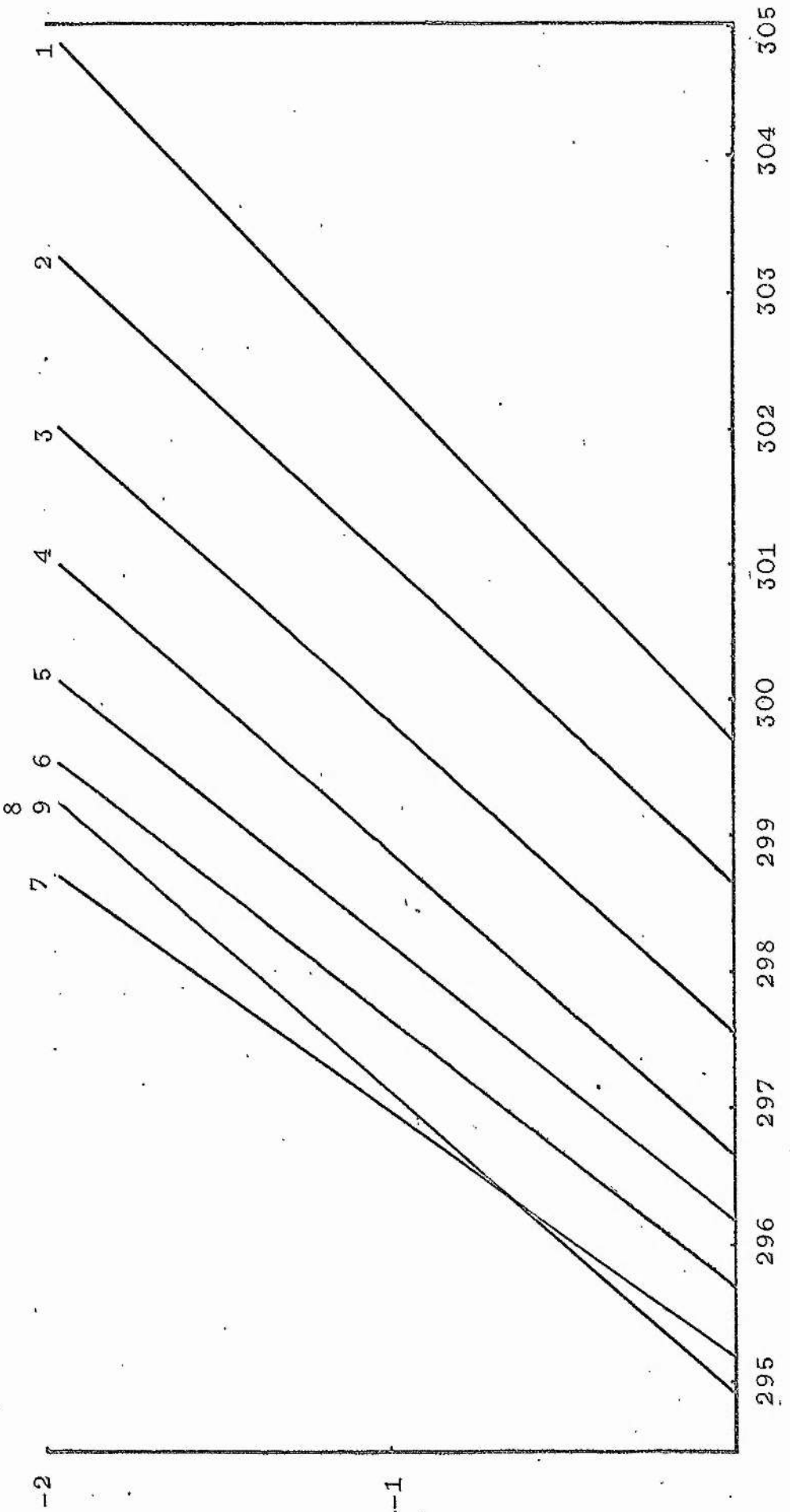
GRAPH 6 - NORMAL

GRAPH 7 - RHEUMATOID

$t_{\frac{1}{2}}$ in minutes. Intercept on $\frac{1}{T}$ axis

is therefore the Ts.

GRAPH 6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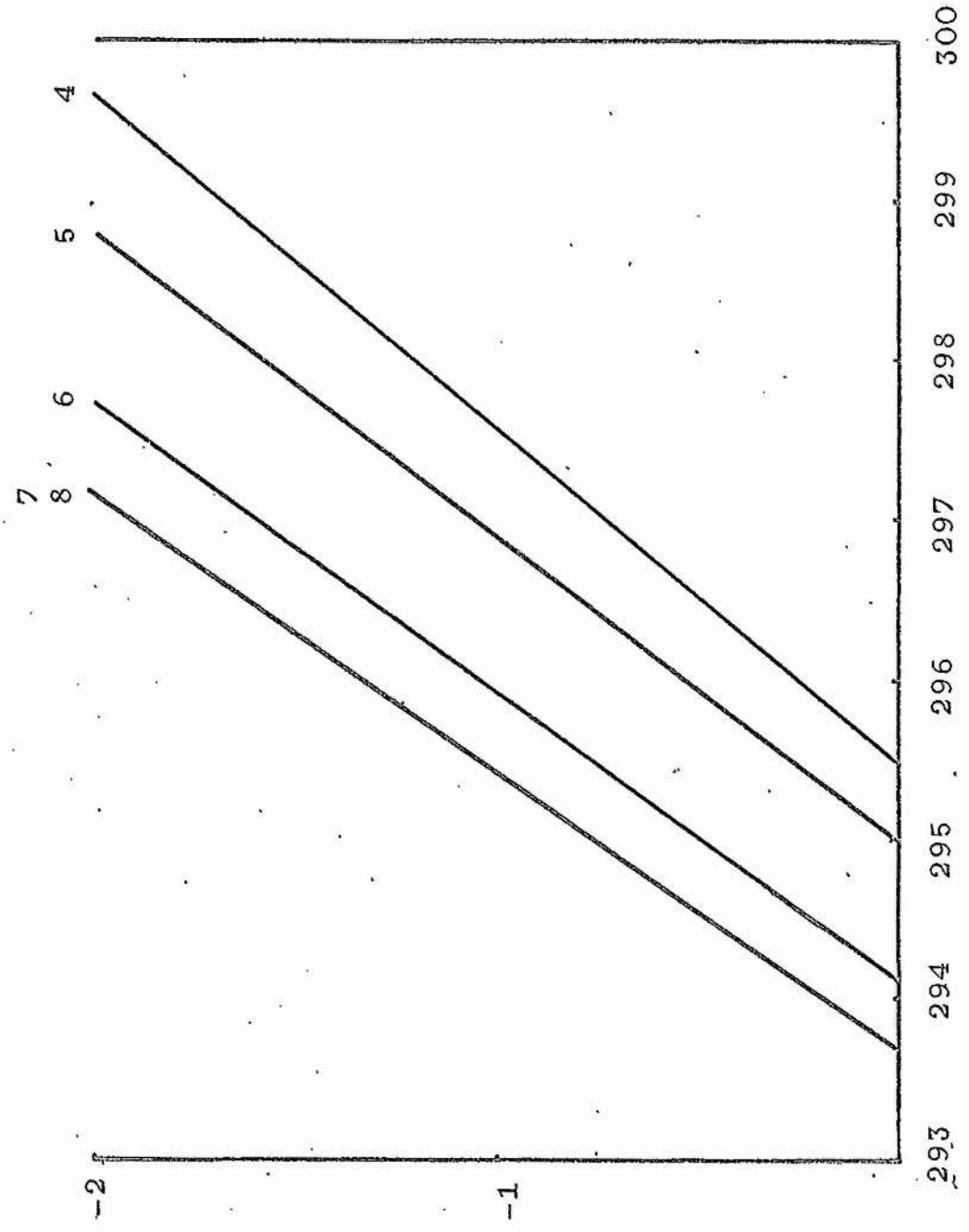
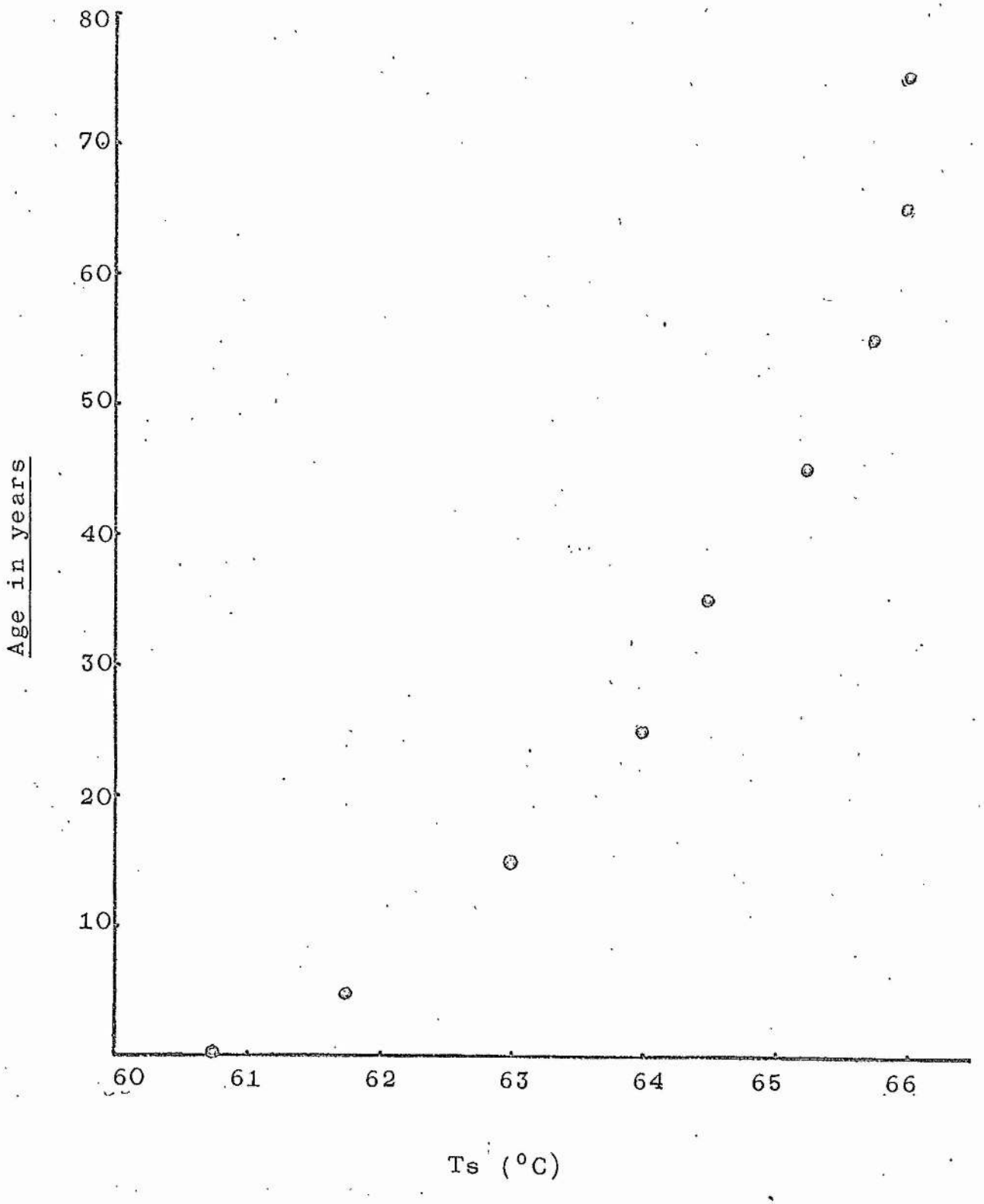
302
303
304
305
$\frac{1}{T} \times 10^5$

GRAPH 7



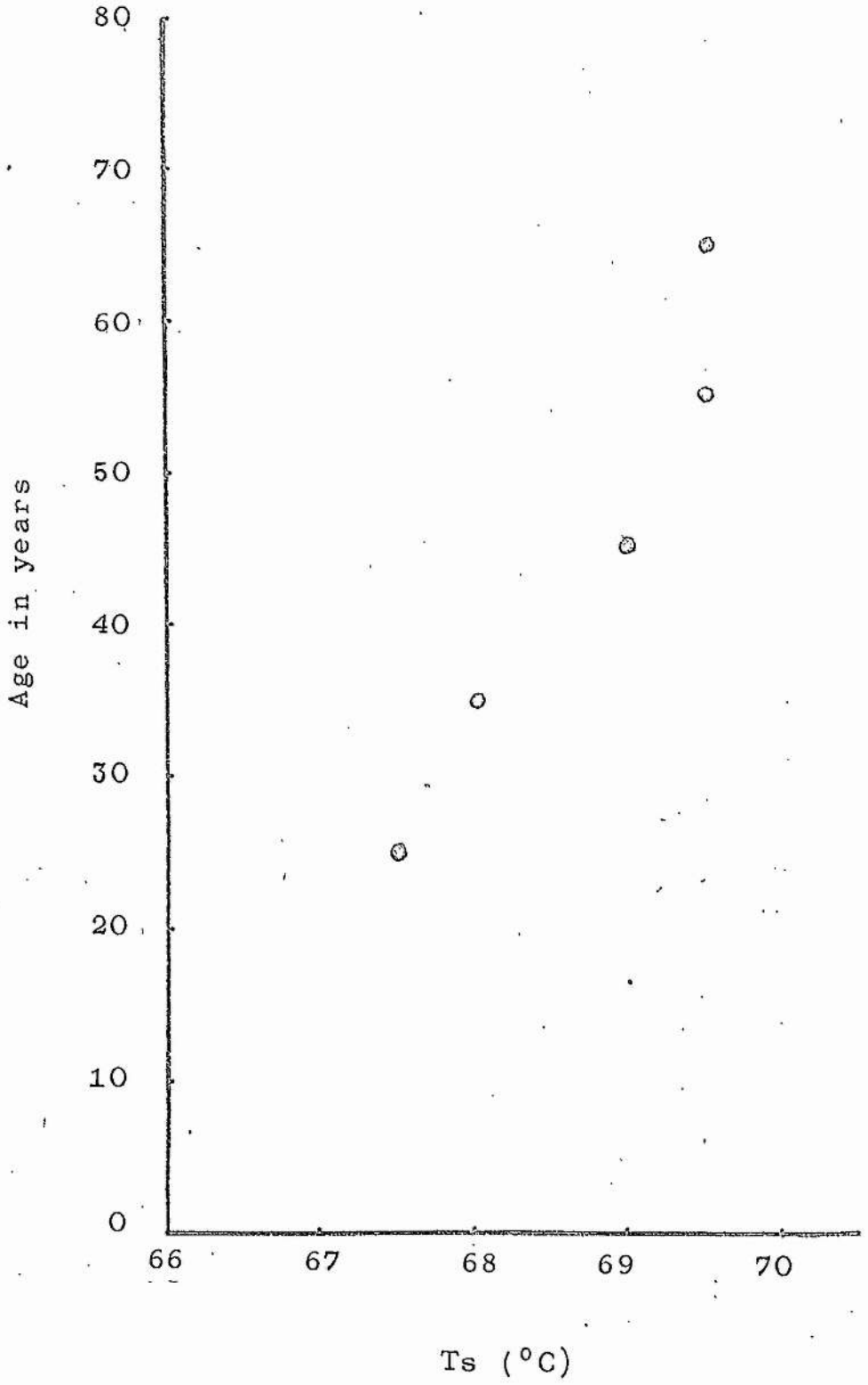
GRAPH 8

Variation of Ts with age in normal human tendon collagen.



GRAPH 9

Variation of Ts with age in rheumatoid human tendon collagen.



The Shrinkage Temperature of Normal and Rheumatoid Human Tendon Collagen after Treatment with α-Amylase.

Collagen fibres which had been precipitated from acid solution after α-amylase treatment of insoluble collagen fibres were used in these studies.

Method.

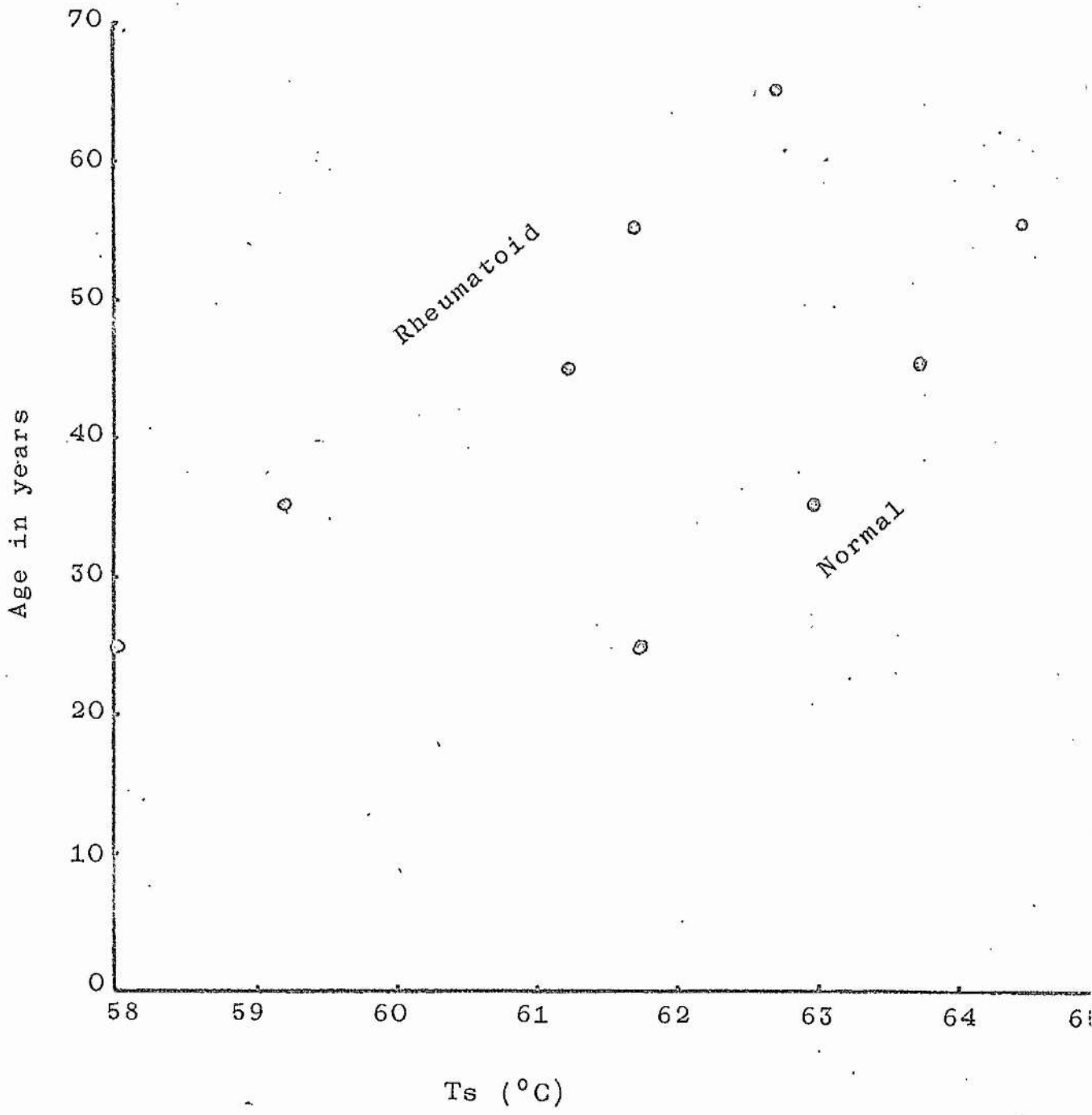
The fibres were thoroughly washed and dialysed in distilled water to remove all traces of salt, acid and enzyme. Determinations of the shrinkage temperature of fibres from different ages of tissue source, both normal and rheumatoid, were carried out as described for previous similar determinations on rat tail tendon collagen and human tendon collagen.

Results.

The variation of the shrinkage temperature with age and disease after α-amylase treatment is presented in Graph 10.

GRAPH 10

Variation of Ts with age in normal and rheumatoid human tendon collagen after α-amylase treatment.



Application of the Arrhenius Equation to the Process of Shrinkage in Normal and Rheumatoid Human Tendon Collagen.

As mentioned in the discussion of Weir's experiments on the application of the Arrhenius equation to kangaroo tail tendon, Akeson went on to suggest the use of these determinations in the assessment of the effects of age and disease on rat collagen. It was therefore considered a possible approach to the assessment of the effects of age and rheumatoid arthritis on human tendon collagen, for, as already indicated there exist differences in the behaviour of these different types of collagen in shrinkage temperature determinations and certain small differences in solubility and chemical properties.

Method.

Values for $t_{\frac{1}{2}}$ and T were obtained from the graphs of $\log \frac{1}{t_{\frac{1}{2}}}$ against $\frac{1}{T}$ used in the determination of shrinkage temperatures of the different types of collagen. These values were used in the plot of:

$$\log \frac{0.6932\ h}{k\ t_{\frac{1}{2}} T} \text{ against } \frac{1}{T}$$ (see Introduction, page 107).

From the linear plots so produced (Graphs 11 and 12) values for ΔH, ΔS and ΔF were determined for each age group of normal and rheumatoid collagens.

ΔH = Slope of Graph x 2.303 x R. (where R is the Gas constant)

$$\Delta S = \frac{\Delta H}{T} - \left(2.303 \times R \times \log \frac{0.6932\ h}{k.T.t_{\frac{1}{2}}} \right)$$

$$\Delta F = \Delta H - T\ \Delta S.$$

GRAPHS 11 & 12

Plots obtained for:

$$\log \frac{0.6932\ h}{k\ T\ t_{\frac{1}{2}}} \quad \text{against} \quad \frac{1}{T}$$

for different age groups, ($t_{\frac{1}{2}}$ in seconds)

1) 0 - 6 months.

2) 7 months to 10 years.

3) 11 - 20 years.

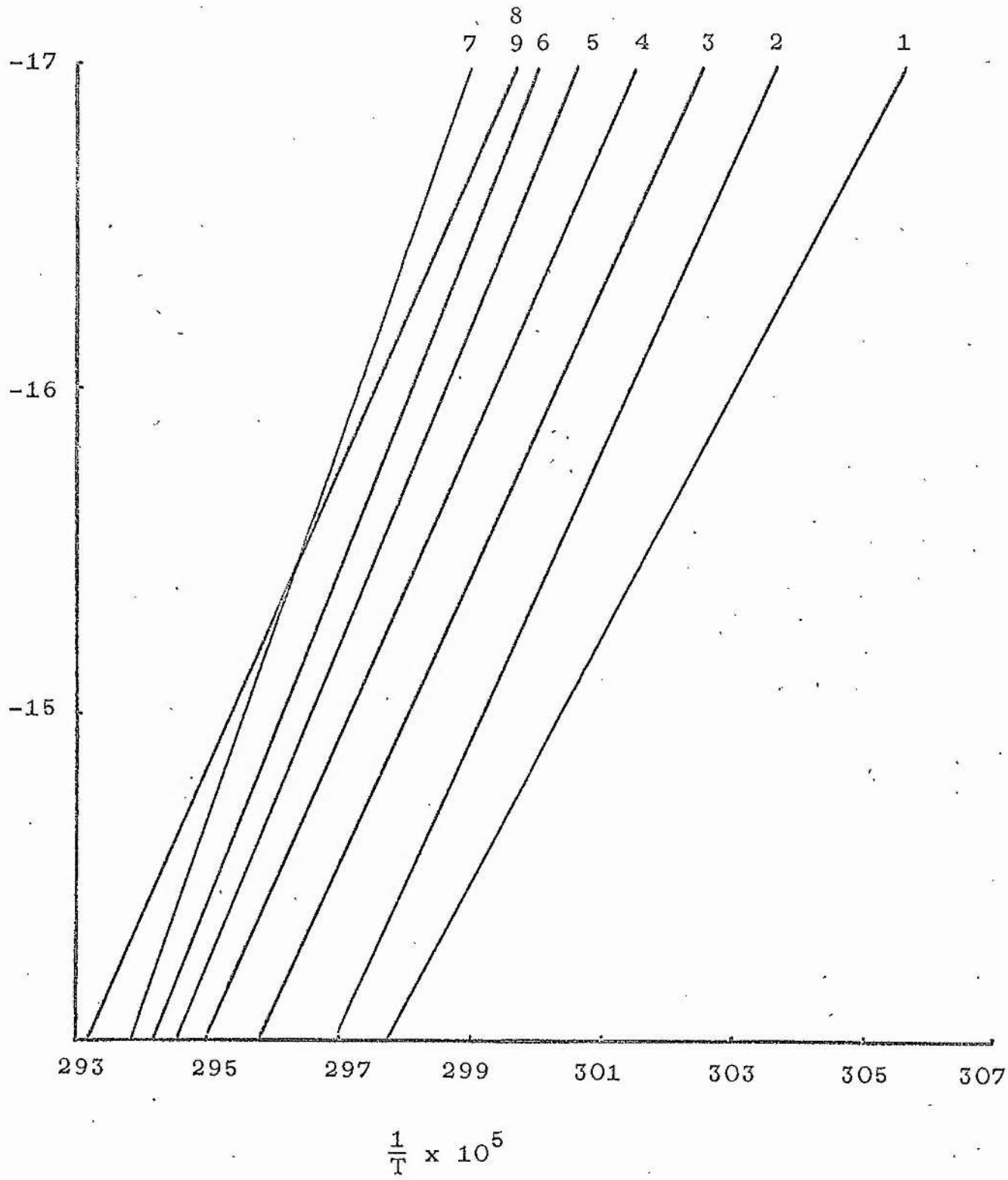
4) 21 - 50 years.

etc.

GRAPH 11 - NORMAL

GRAPH 12 - RHEUMATOID

GRAPH 11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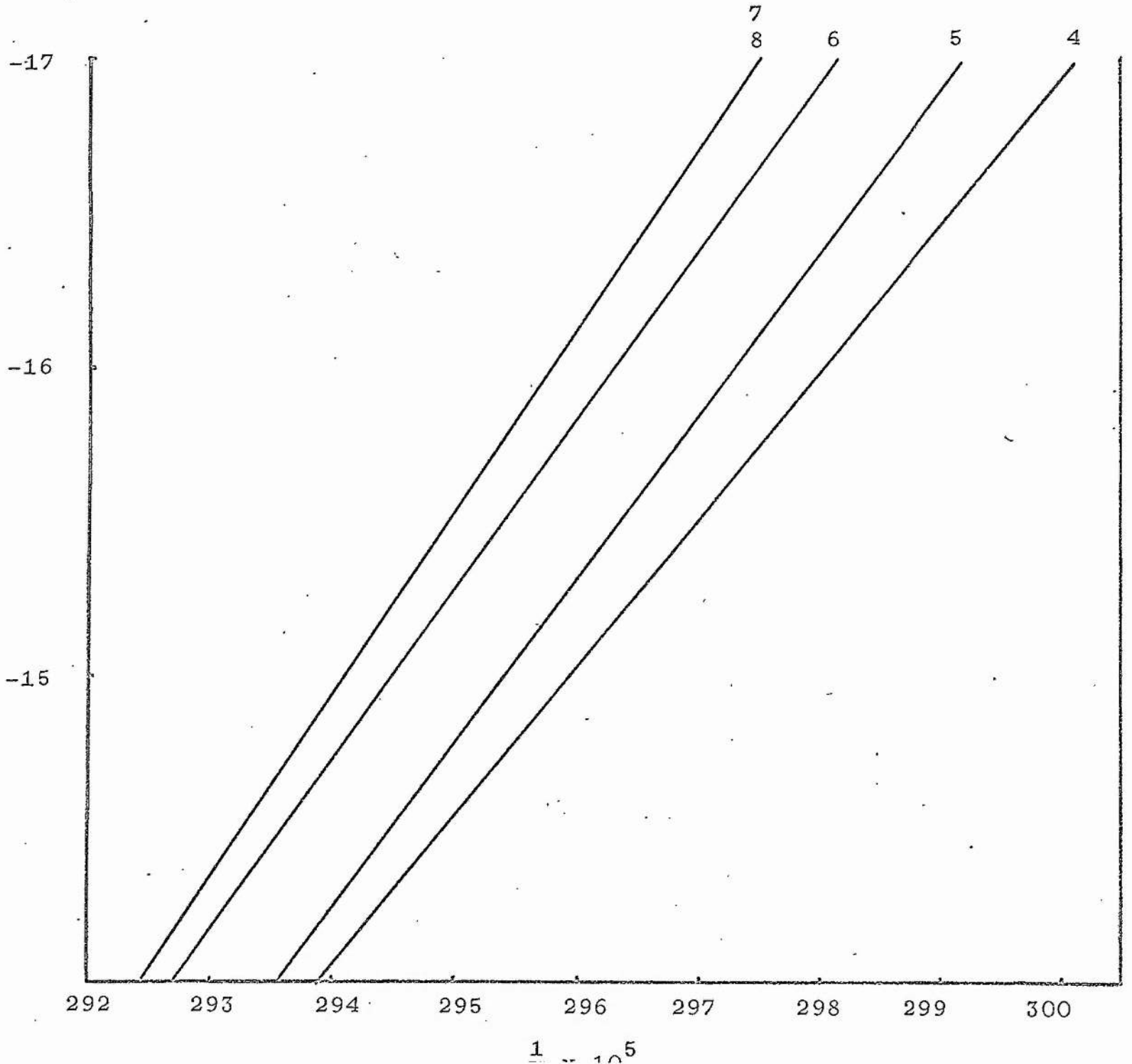
$\frac{1}{T} \times 10^5$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$\frac{1}{T} \times 10^5$

GRAPH 12

In the determination of ΔF a value of 331 ($58^{o}C$) was used for T in order to obtain comparative values for this parameter.

Results.

The results are presented in tabular form (Table 7). Also presented are values for normal and rheumatoid collagen after α-amylase treatment obtained in a similar manner. (Table 8

TABLE 7.

The Values of ΔH, ΔS and ΔF for the Shrinkage Process of Norma and Rheumatoid Tendon Collagen and the Effect of Age of the Tissu Source.

Age	T_s (°C) (1 min.)	ΔH (kcal/mole)	ΔS (cal/mole deg.)	ΔF_{58} (kcal/mole)
Normal				
0-6 mths.	60.75	173.9	453.13	23.91
7 mths-10 yrs.	61.75	199.1	527.23	24.59
11-20 yrs.	63.0	205.0	542.63	25.34
21-30 yrs.	64.0	211.0	558.63	26.09
31-40 yrs.	64.5	223.2	593.63	26.71
41-50 yrs.	65.25	232.5	619.63	27.34
51-60 yrs.	65.75	259.5	698.53	28.29
61-70 yrs.	66.0	209.6	550.53	27.37
71-80 yrs.	66.0	209.6	550.53	27.37
Rheumatoid				
21-30 yrs.	65.5	217.8	576.03	27.13
31-40 yrs.	66.0	245.3	656.03	28.15
41-50 yrs.	67.0	254.5	680.93	29.11
51-60 yrs.	67.5	271.4	729.73	29.86
61-70 yrs.	67.5	271.4	729.73	29.86

TABLE 8

The Values of ΔH, ΔS and ΔF for the Shrinkage Process of Normal and Rheumatoid Tendon Collagen after Treatment with α-Amylase and the Effect of Age of the Tissue Source.

Age	T_s (°C) (1 min.)	ΔH (kcal/mole)	ΔS (cal/mole deg.)	ΔF_{58} (kcal/mole
Normal				
21 - 30 yrs.	61.75	212.8	568.03	24.78
31 - 40 yrs.	63.0	209.6	556.23	25.49
41 - 50 yrs.	63.75	217.8	579.33	26.04
51 - 60 yrs.	64.5	217.8	577.23	26.54
61 - 70 yrs.	65.0	208.2	548.43	26.67
Rheumatoid				
21 - 30 yrs.	58.0	189.1	485.53	22.39
31 - 40 yrs.	59.25	199.1	531.83	23.06
41 - 50 yrs.	61.25	200.4	531.93	24.33
51 - 60 yrs.	61.75	206.4	549.13	24.63
61 - 70 yrs.	62.75	206.4	547.33	25.23

The Effect of Some Anti-rheumatic (Anti-inflammatory) Drugs on the Shrinkage Temperature of Normal and Rheumatoid Human Tendon Collagen from Tissue Sources of Different Ages.

Collagen fibres were prepared from normal and rheumatoid tissue sources as described previously. The drugs used were chosen for their common and present day usage in the treatment of rheumatoid arthritis. These drugs are listed below with their source, and, as far as could be ascertained, their range of dosage when used in the treatment of this disease.

Cortisone Acetate (Cortelan); Glaxo; Dosage 50 - 200 mg. per day.

Co-K-Sal, supplied as tablets each containing 325 mg. Acetyl-salicylic acid, 20 mg. Ascorbic acid, and 0.35 mg. Aceto-monaphthone. Paine and Byrne Ltd.; Dosage 1 - 3 tablets every 3 hrs.

Prednisone (1.2-dehydro-cortisone); Parke and Davis; Dosage 5 - 20 mg. per day.

The effect of Gold was also determined. This metal is used in the form of Sodium-Auro-Thiomalate for treatment of rheumatoid arthritis. The dosage level is usually about 10 mg. increasing to 100 mg. daily at 5 - 7 day intervals. Gold chloride; Johnson's, Hendon; was used in these experiments.

Method.

Solutions of the drugs were made up in distilled water to the concentrations listed below. In some cases the drug was partially insoluble and the residue was then filtered off. The concentrations were assumed to approximate a tissue (physiological

concentration on an average daily dosage rate.

Cortisone Acetate; 30 mg. per litre.

Co-K-Sal; 30 mg. Acetyl Salicylic acid per 100 ml.

Prednisone; 3 mg. per litre.

Gold chloride; 10 mg. gold per litre.

Collagen fibres were soaked in the drug solutions for periods of 24 hrs. at room temperature ($18^{\circ}C$). The fibres were then thoroughly washed and dialysed in distilled water to remove all the drug solution.

Shrinkage temperature determinations were carried out exactly as described previously, using Weir's method.

Results.

The results are presented in Table 9. The effects of these drugs on the shrinkage temperature of α-amylase treated normal and rheumatoid collagen are also shown for comparison (Table 10).

TABLE 9

The Effect of some Drugs on the Shrinkage Temperature of Normal and Rheumatoid Human Tendon Collagen from Tissue Sources of Different Ages.

Age	T_s (°C) (1 min.)	T_s (°C) after treatment with:			
Normal	Original	Ce-K-Sal	Prednisone	Cortisone Acetate	Gold chloride
21 - 30 yrs.	64.0	63.75	66.75	64.5	69.0
31 - 40 yrs.	64.5	63.75	67.0	64.75	70.0
41 - 50 yrs.	65.25	65.25	67.0	65.75	70.0
51 - 60 yrs.	65.75	65.5	67.75	66.25	71.0
61 - 70 yrs.	66.0	66.0	68.0	66.0	71.0
Rheumatoid					
21 - 30 yrs.	65.5	65.25	66.5	65.75	70.0
31 - 40 yrs.	66.0	65.75	67.25	66.25	71.0
41 - 50 yrs.	67.0	67.0	68.0	67.0	71.0
51 - 60 yrs.	67.5	67.25	68.25	67.5	72.0
61 - 70 yrs.	67.5	67.5	68.5	67.75	72.0

TABLE 10.

The Effect of some Drugs on the Shrinkage Temperature of Normal and Rheumatoid Human Tendon Collagen after Treatment with α-Amylase.

Age	T_s (°C) (1 min.)	T_s (°C) after treatment with:			
Normal	Original	Ce-K-Sal	Prednisone	Cortisone Acetate	Gold chloride
21 - 30 yrs.	61.75	60.0	63.75	62.5	70.0
31 - 40 yrs.	63.0	61.0	65.25	64.0	69.0
41 - 50 yrs.	63.75	62.0	65.0	64.5	68.0
51 - 60 yrs.	64.5	63.75	67.0	65.25	70.0
61 - 70 yrs.	65.0	65.25	66.75	65.75	72.0
Rheumatoid					
21 - 30 yrs.	58.0	48.0	60.0	59.0	68.0
31 - 40 yrs.	59.25	48.5	62.0	60.0	70.0
41 - 50 yrs.	61.25	55.5	63.0	62.0	70.0
51 - 60 yrs.	61.75	58.75	63.5	62.25	69.0
61 - 70 yrs.	62.75	62.25	65.5	63.0	70.0

7) The Treatment of Normal and Rheumatoid Human Tendon Collagen with Alkali.

It has been shown (Hey and Stainsby, 1965) that the solubility of collagen from skin, tendon or ossein was enhanced by treatment of the tissue with alkali at low temperatures. The effect was suggested to be due to a breaking of inter-molecular types of cross-links.

This method was used to obtain a soluble form of collagen from the insoluble normal and rheumatoid human tendon collagen used in the previous studies.

Method

Separate samples of collagen fibres derived from normal and rheumatoid tendons of different ages were swollen in 2N. Sodium Hydroxide (12 ml./g. collagen as dry protein) for 6 days at 3°C. Precooled acetic acid was then added, with thorough stirring, in sufficient concentration to lower the acidity of the suspension to about pH 3. The temperature of the acidified suspension was allowed to rise to about 18°C. (room temperature) and it was stirred at this temperature for 48 hrs. The residue was removed after centrifugation at 35,000 g., and re-extracted with 0.2M. acetic acid. For each collagen sample the two extracts were combined and further studies carried out on these clear viscous solutions.

The Ultra-Violet Absorption Spectra of Acetic Acid Extracts of Alkali Treated Normal and Rheumatoid Human Tendon Collagen.

Solutions of alkali treated normal and rheumatoid collagens (pH 3) were scanned over the wavelength range 200-450 mμ. using 1 cm. cuvettes in a Unicam SP 800.

Results

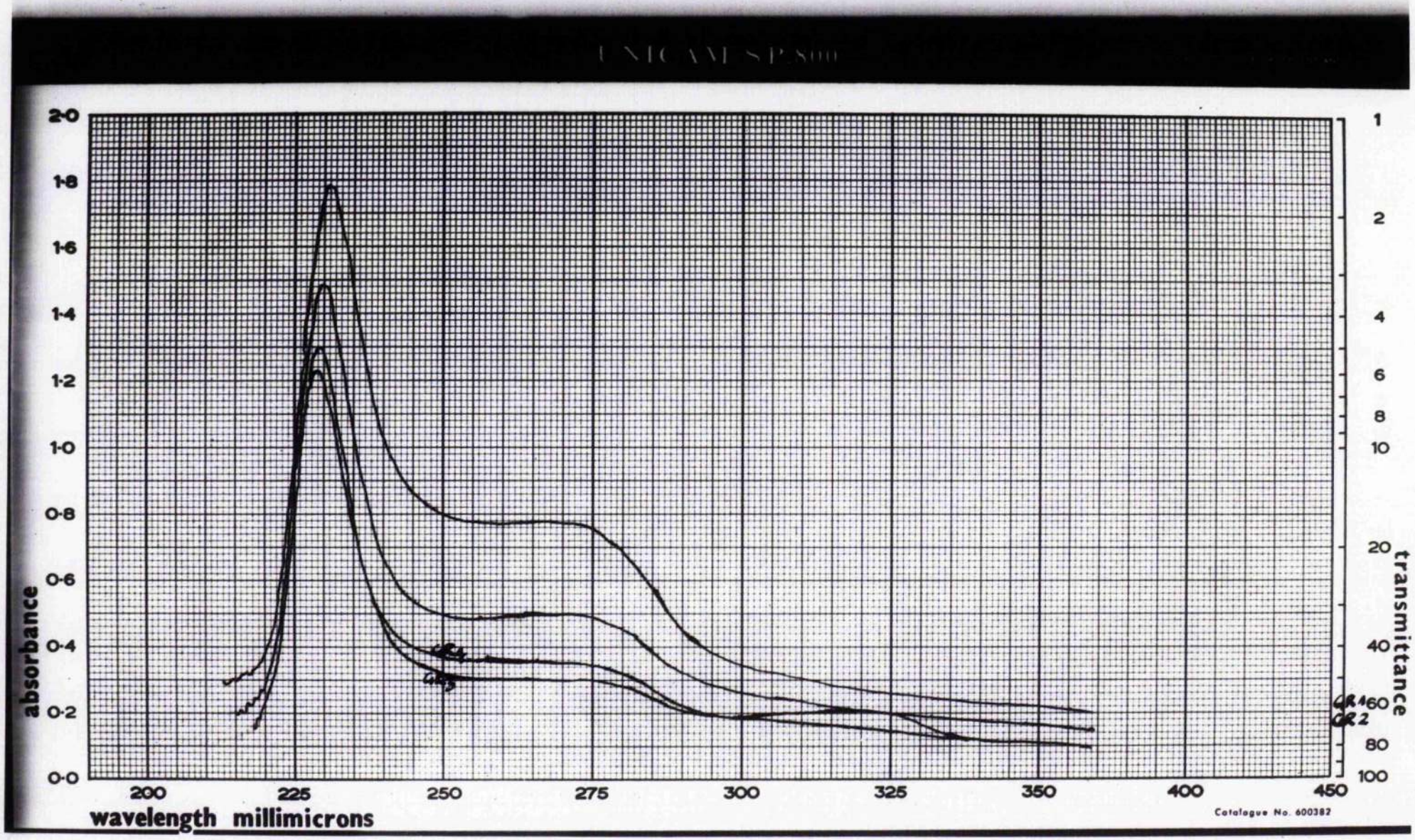
Representative spectra are presented in Plates V-VII. No age differences were apparent but it can be seen that the rheumatoid samples exhibited a peak at 275 mμ which was absent in the majority of spectra from normal samples.

PLATES V - VII

Plate V : U.V. Spectra of Normal Collagen.

Plate VI : U.V. Spectra of Rheumatoid Collagen.

Plate VII : U.V. Spectra of Two Normal and Two "Rheumatoid" Collagens.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0·0
transmittance
1
2
4
6
8
10
20
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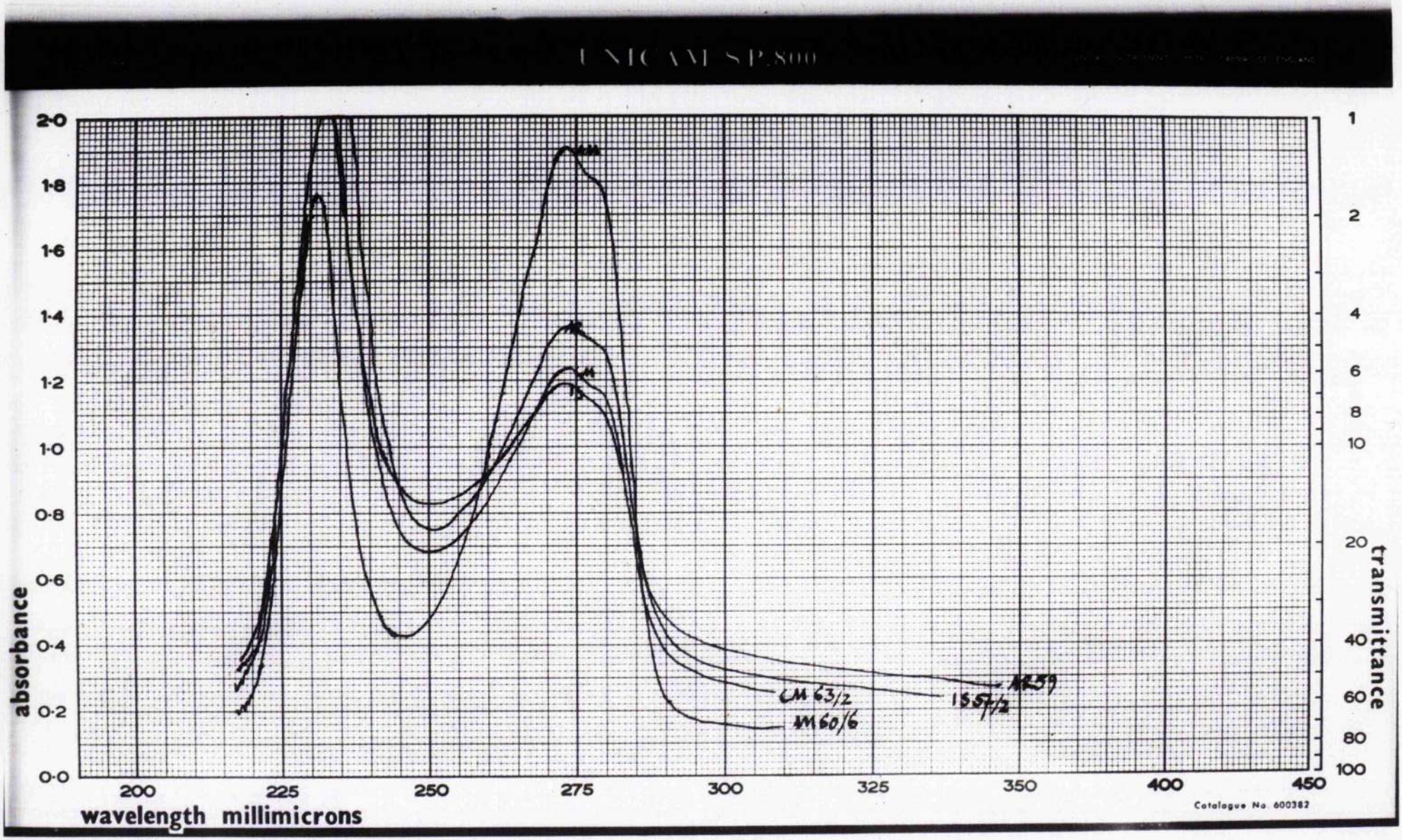
wavelength millimicrons
GR1
GR2
Catalogue No. 600382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0·2
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transmittance
1
2
4
6
8
10
20
40
60
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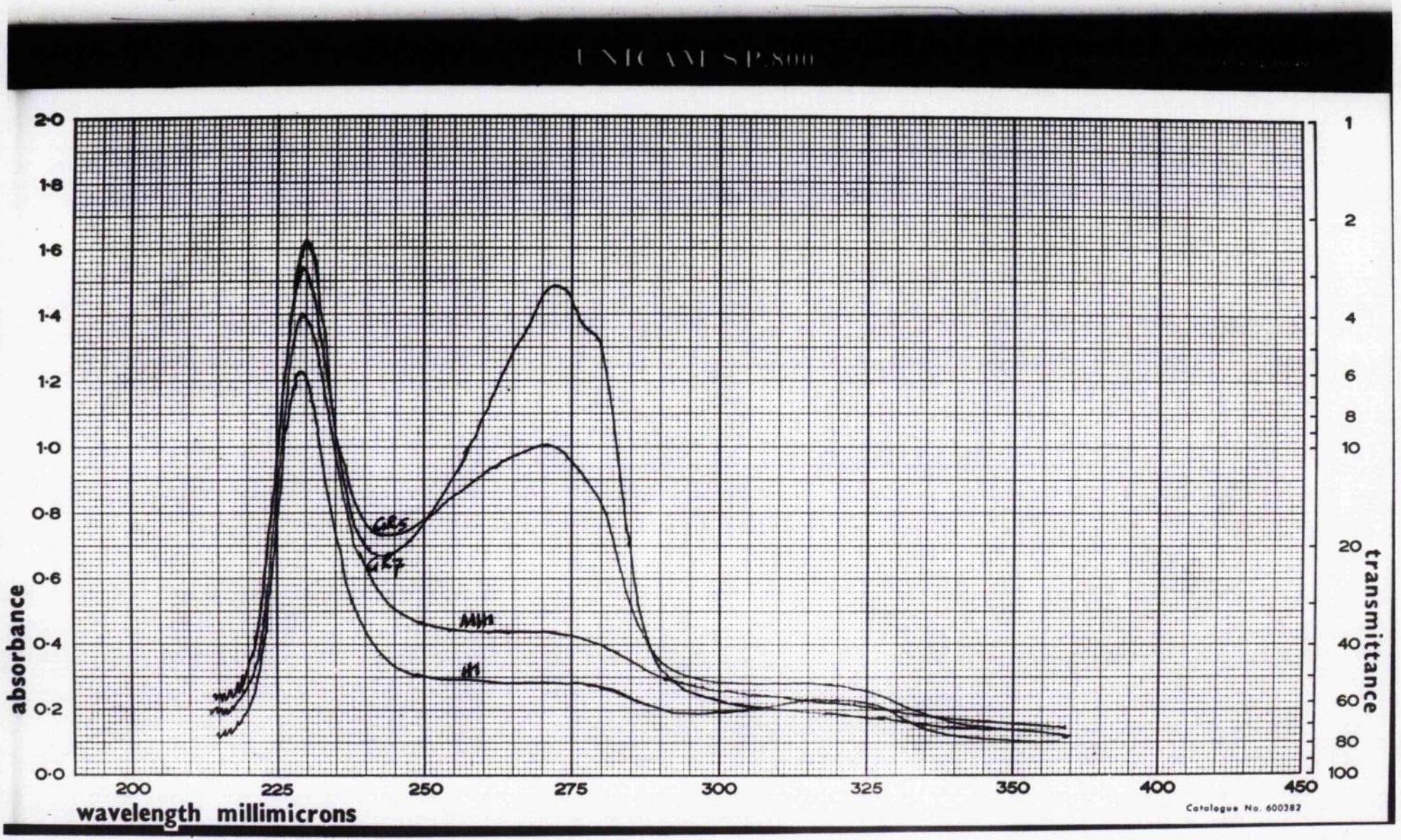
Catalogue No. 600382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absorbance
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transmittance
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225
250
275
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325
350
400
450
wavelength millimicrons
Catalogue No. 600382

8) The Electron Microscopic Examination of Normal and Rheumatoid Human Tendon Collagen Fibres.

Fibres of normal and rheumatoid collagen used in the previously mentioned solubility and shrinkage temperature studies, and fibres precipitated from acetic acid solution after alkali treatment were finely divided by vigorous homogenisation in distilled water. Electron microscope grids (carbon filmed) were carefully layered with these suspensions and dried in a desiccator over anhydrous calcium chloride. The grids were then stained with Phospho-tungstic Acid pH 4.2 (positive staining) and again dried completely before examination in the electron microscope. (The microscope used was an AEI 6B model.

Representative electron micrographs are presented in Plates VIII-XI.

PLATES VIII - XI

Plate VIII : Electron Micrograph of Collagen Fibres from Normal Human Tendon;
Magnification : 20,000 x 2.91.
Periodicity : $694 \pm 24 \times 10^{-8}$ cm.

Plate IX : As Plate VIII;
Periodicity : $668 \pm 25 \times 10^{-8}$ cm.

Plate X : Electron Micrograph of Collagen Fibres from Rheumatoid Human Tendon;
Magnification : 20,000 x 2.91.
Periodicity : $667 \pm 24 \times 10^{-8}$ cm.

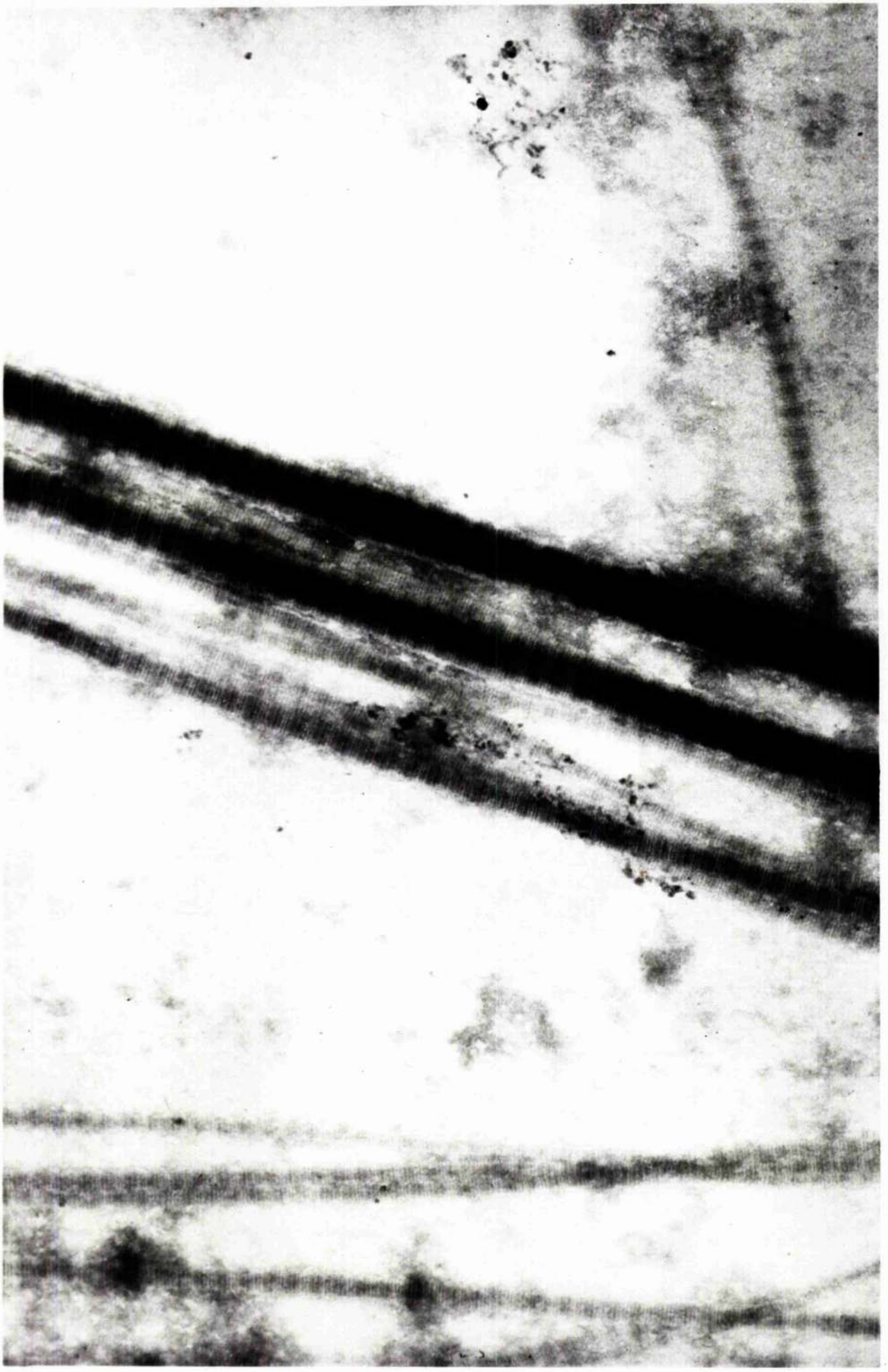
Plate XI : Electron Micrograph of Collagen Fibres from Rheumatoid Human Tendon after Alkali Treatment;
Magnification : 20,000 x 2.91.
Periodicity : $614 \pm 20 \times 10^{-8}$ cm.

24AB2

20,000 x 2.91

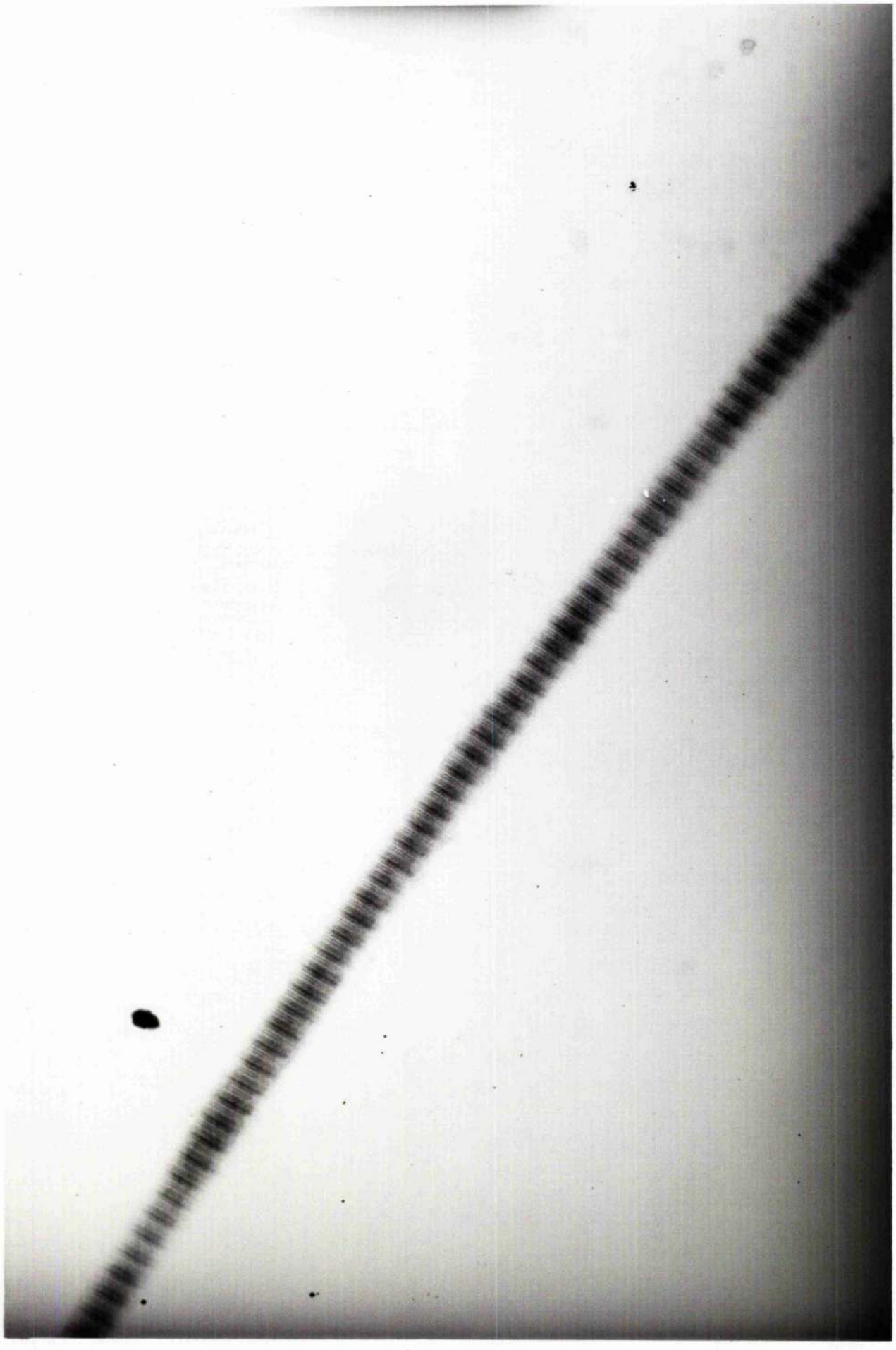
24 A 15 3

20,000 x 2.91



GLAC

20,000 x 291



2 7KA2

40,000×∠91

DISCUSSION.

Introductory

In considering the effects of rheumatoid arthritis on tendon collagen it is necessary, as already intimated, to take into account the effects of age on the tissue source. For this reason almost equal emphasis has been placed on variations in collagen properties due to both age and disease in this series of studies.

It must also be borne in mind that in such a series of studies the older the tissue source used for a supply of normal collagen to provide control values the more likely it is that some effects due to unconfirmed, or even undiagnosed, connective tissue disease might be present.

Although there was slightly more variation in the stability of rheumatoid collagen at different ages as seen on comparison of graphs 8 and 9 it was apparent that the normal controls used in these studies were in almost all cases free of any involvement with rheumatoid arthritis. However, two samples previously taken as normal did show abnormal shrinkage temperatures and absorption spectra. The significance of this is discussed under the appropriate headings.

1) Analytical Results.

The amino acid composition of the normal and rheumatoid human tendon collagen used as a starting material for experimental work presented in this thesis, agreed well with accepted values for collagens in general (Table 2 and Table 6). A direct comparison with those values published by Eastoe (1955) for an acid extract of human tendon indicated that the main differences consisted in rather high proline and rather low hydroxyproline values.

In the comparative determinations presented in Tables 4 and 5 some variations were observed which could be due to either aging effects or disease effects. However, the values found for Total Nitrogen, Ash and Hexosamine contents of both types of collagen before and after α-amylase treatment agreed with literature values, (see Grassmann et al., 1957a, Tristram and Smith 1963).

The Moisture content and Hydroxyproline content showed variations which were related to age of the tissue source and to the presence of rheumatoid arthritis.

In the normal collagen the moisture content drops with increased age, to a value which was 58% of the content for the youngest age group. In the rheumatoid collagen the moisture content was lower than that of the equivalently aged normal collagen although the differences were not so marked in the older age groups. Over the age range available the moisture content of rheumatoid collagen dropped to a value

which was 71% of the content for the youngest age group. The equivalent decrease in the normal collagen over the same age range resulted in a value which was 59% of the content for the 21-30 age group. After α-amylase treatment the moisture content of both types of collagen was increased at any particular age. A decrease in this content was still apparent with increasing age although the overall decrease in the normal collagen was reduced, the value at the highest age being 70% of that for the youngest age group.

A decrease in the moisture content of whole tissue during increasing age has been documented by several authors (see Verzar 1957b, Grant 1963). The process has been ascribed to two possible factors:

1) The general decrease in the mucopolysaccharide content of the tissue during aging, these polymers having a high water binding capacity.

2) An increase in the cross-linking density of the collagen component, leading to a less porous structure, (Hall and Kinoshita 1964).

Both factors may be involved in whole tissue but in the system dealt with in those studies the second factor was the more probable. Thus it could be proposed that there was an increase in cross-linking of the normal collagen with increasing age of the tissue source and also that rheumatoid collagen (from human tendon) was more cross-linked than normal collagen of an equivalent age.

The hydroxyproline content of normal collagen showed a variation which indicated a tendency for the content of this amino acid to rise with increasing age of the tissue source. However no such conclusion could be drawn from the results for rheumatoid collagen. It was found, in fact, that the hydroxyproline content of one sample of rheumatoid collagen in the 61-70 age group was as low as 4.5%. This observation, considered along with the other values recorded for rheumatoid collagen, might suggest an increasing contamination of this type of collagen, with increasing age, by some other protein containing little, or no, hydroxyproline. The effect of α-amylase treatment appeared to be that of increasing the hydroxyproline content of both types of collagen to a small extent.

The significance of the variation of hydroxyproline content in the normal collagen will be discussed in relation to the shrinkage temperature studies, (page 151).

The significance of the effects of α-amylase treatment on the moisture, hydroxyproline and hexose content of normal and rheumatoid collagen cannot be explained simply. As already stated this enzyme has been used by some authors to pretreat insoluble collagen in order to obtain a more soluble form. It has been assumed on the basis of very little evidence that the enzyme had no proteolytic activity towards collagen or gelatin. From this series of studies it would appear that its main action was on the hexose content of collagen. The hexose content of aged normal human collagen

before and after α-amylase treatment agrees well with the results of other authors (Steer 1966, gave a value of 0.7% hexose in insoluble collagen and 0.36% in amylase solubilised collagen), however, similar values for young collagen were not available for comparison.

The overall effect in the case of normal collagen seemed to be one of a decreasing susceptibility of the hexose to α-amylase attack with increasing age. With rheumatoid collagen however, the effect of the enzyme was of a similar magnitude to that which it had on the younger normal age groups, and moreover the age of the rheumatoid collagen had little effect, if any, on the extent of this action.

These results might be interpreted as indicating an increasing involvement of hexose in some type of linkage in progressively older normal collagen. On the other hand it could also be said that the hexose became less available in the older age groups due purely to steric factors. Some work presented by Hörmann (1966) might serve to clarify this situation. He showed that the action of α-amylase on soluble collagen resulted in the liberation of hexose reducing groups. Similar treatment of insoluble collagen, however, only resulted in the liberation of reducing groups if the collagen had been previously treated with hydroxylamine. It was suggested that α-amylase splits the O-glycosidic linkage in soluble collagen but in insoluble collagen a second (ester) type of bond was present which was responsible for the inability

of α-amylase to release the reducing groups in this mature type of collagen. In support of this theory was the evidence of Bello (1960), Hörmann (1960a) and Joseph and Bose (1962), previously mentioned in the introduction to this thesis, for an increase in the ester type cross-links in collagen from progressively older tissue sources. That about 0.4% hexose was an integral part of the collagen molecule has been shown by Hörmann and Fries (1958) and furthermore, Blumenfeld et al., (1963) have demonstrated that the hexose molecules might be bound solely by carbon 1 in ichthyocol, a protein in which no extensive cross-linking is apparent. Thus these results support, indirectly, the increased involvement of hexose in cross-links of the ester type during aging.

Bearing this discussion in mind it would appear that in rheumatoid collagen the involvement of hexose in cross-links during the aging process was in some way suppressed. This agreed with the suggestion made by Steven (1966a) that a hydroxylamine sensitive alkali-stable type of cross-link existed in normal collagen which was absent in rheumatoid collagen, but it will be noticed that this was apparent only in collagen from tissue sources over the age of 51 yrs.

There is evidence available (Steer 1966) that the preparation of α-amylase used in these studies, and also used by Steven (1964a), had a demonstrable proteolytic activity on collagen. It has also been demonstrated (Steer 1966) that after α-amylase treatment only α components can be found in

the subsequently fractionated collagen. This latter observation, plus the slight increase in hydroxyproline content after ∝-amylase treatment shown in these studies, indicated that the proteolytic activity was directed to attack of terminal non-helical regions of the molecule. Further aspects of the effects of ∝-amylase will be discussed with reference to its alteration of the shrinkage temperature of collagen (page 156), its action cannot be assumed to be completely non-proteolytic.

2) Solubility Studies.

In agreement with the results mentioned in the introduction (page 59), these studies indicate that as collagen matures it becomes more insoluble in acetic acid or citrate buffer (pH 3.8). Rheumatoid collagen appeared to be more insoluble than normal collagen over the age range available. The inference could be that there was an increase in cross-link density as the collagen matured and that rheumatoid collagen was comparatively more cross-linked, at any age, than the normal collagen. These results agreed with those of Bakerman (1965) although the decrease in solubility with age was not so marked.

The effects of hyaluronidase, trypsin or papain on the solubility of the two types of collagen was as expected from their recognised modes of action. Only in the case of trypsin treatment was the solubility increased to any extent. However the overall changes with age are similar before and after enzyme

treatment and the rheumatoid collagen remained less soluble than the normal.

It was interesting to note a slight effect of age on the extent of trypsin action in terms of hydroxyproline release. If the action of trypsin on collagen is solely to attack the telopeptide region, as suggested by many authors, then these results might suggest an increase in the number of cross-links present in this region with increasing age, in agreement with Steven (1965a). However, the quantity of hydroxyproline released would appear to be slightly higher than that expected from breakdown of telopeptides which are assumed to be almost devoid of this amino acid, (Rubin et al., 1963).

3) Shrinkage Temperature Studies.

The use of a range of temperature over which shrinkage occurs, or the temperature of incipient contraction, as values for the shrinkage temperature of collagen has led to an acceptance of these values as easily determined indications of the structural stability of collagen from different sources. However their true comparative value is in question if one accepts shrinkage as a rate process. From the data presented by Weir (1949) and Weir and Carter (1950) it would appear that shrinkage must be interpreted as a rate process and, as such, a description of the shrinkage temperature as the temperature at which shrinkage to half length occurs in one minute would lead to more easily comparable and, in the opinion of this author, more significant results. As shown by Weir and Carter a

thermodynamic interpretation can be carried out using the information derived from this type of shrinkage temperature determination.

The method as used in these studies gave values for the shrinkage temperature of rat tail tendon collagen and normal human tendon collagen which lay within the range of temperature generally quoted for these values by other authors. (Preliminary studies were carried out on rat tail tendon to confirm that the method was usable).

In the results presented (Graphs 8 and 9) the largest difference in the shrinkage temperature of duplicate samples of collagen from the same age source was 0.2°C. The range of shrinkage temperature in any age group of human tendon collagen was about 0.5°C. above and below the average value in almost all cases although in the older age groups and in the rheumatoid samples the range was smaller than this.

The use of freely suspended collagen fibres has recently been shown to be acceptable for reproducible determinations by Naghski, Wisnewski, Harris and Witnauer (1966) in their studies on the correlation of differential thermal analysis data with the shrinkage temperature of collagen and leather.

Values for the shrinkage temperature of human collagen (from fascia lata) and the variation with age were published by Brown et al., (1958). These were expressed as the range of temperature over which the collagen shrank and are presented

below for comparison.

Age	Shrinkage range
Foetal age group	54.4 - 60.0°C.
0 - 14 yrs.	57.7 - 63.9°C.
15 - 25 yrs.	59.8 - 65.7°C.
26 - 44 yrs.	59.1 - 66.2°C.
45 - 88 yrs.	61.2 - 67.0°C.

Stringer and Highton (1960) published a range of normal and abnormal skin collagen shrinkage temperatures, expressed as the temperature of incipient contraction, which extended over the range 57 - 65°C. No specific mention was made of age although it was suggested that "normal" human skin collagen had a shrinkage temperature of 63°C. and young human skin collagen had a lower shrinkage temperature and was classed as a variant from the normal.

It can be seen therefore that the shrinkage temperature values presented in this thesis were in good agreement with those values in the literature, and that the variation of shrinkage temperature with age was also of the same order as previously published values. However few, if any, references seemed to exist in which work of this type has been carried out on human tendon collagen, although, fascia lata in some aspects could be considered as a tendon particularly the distal portion near the knee joint.

With reference to the shrinkage temperature of

rheumatoid tendon collagen as far as could be ascertained an increase in the shrinkage temperature over that of normal tendon collagen of the same age has not been reported previously. Decreases in the shrinkage temperature of collagen from rheumatoid nodules have been reported by both Brown et al., (1958) and Stringer and Highton (1960). The former authors determined the shrinkage temperature of collagen from the three separate areas of the nodule, whereas the latter authors used collagen from the whole nodule in their determinations.

	Capsular region	Tough fibrous region	Yellow necrotic region
Brown et al., (1958) Ts values for nodular collagen from older patients.	58 - 64°C	51 - 61°C	54 - 64°C
	60 - 66°C	52 - 64°C	55 - 60°C
	60 - 67°C	53 - 61°C	55 - 65°C
Stringer and Highton (1960)	Ts of nodular collagen at the lower end of their 57-65°C range		

Both these groups of authors have also published shrinkage temperatures for collagen from other tissues of rheumatoid arthritis patients. Stringer and Highton quoted a value for abnormal skin collagen of 62°C. and Brown et al., quoted the shrinkage temperature ranges shown below for collagen from fascia of three age groups of rheumatoid arthritis patients.

Age	Shrinkage range
0 - 14 yrs.	58 - 66°C.
26 - 44 yrs.	58.5 - 66°C.
45 and above.	59 - 67°C.

In comparison therefore the results presented by Brown et al., for rheumatoid fascia only showed a variation in the lower values of the shrinkage range in the two older groups. The youngest age group in fact showed a slight rise in the shrinkage range in comparison with the normal values.

It was pointed out early in this century (Powarnin and Powarnin and Aggeew, quoted by Gustavson 1956) that the shrinkage temperature of leather was an indication of the tanning effect. For example, the "boiling test" has been used for many years as standard tannery procedure. However it was not until 1929 that it was suggested, by Meyer, that the tanning agents were cross-linking the protein chains.

As already mentioned in the introduction the contraction of the collagen fibre, brought about by the application of heat, is generally considered to result from the weakening or dislocation of the inter-chain hydrogen bonds. According to Gustavson (1956) these bonds might be of two types, those linking peptide bonds on different chains, and those between peptide bonds and side chain hydroxyl functions. However, some contribution to stability must also be due to electrostatic links and the different types of covalent link already discussed. Any increase in the number of the hydrogen bonds

or in the number or strength of the electrostatic and covalent links has been shown to lead to an increase in the temperature required to cause shrinkage, (see for example the comprehensive study by Lennox 1949).

A comparison of the shrinkage temperature data for normal collagen presented here and the analytical results suggests that the increased stability of the protein during aging could be due to a combination of two processes.

a) An increase in hydroxyproline content which was originally suggested to influence the stability of collagen due to increased hydrogen bonding by Gustavson (1955c).

b) An increase in covalent (ester) type bonds in agreement with the findings of other authors previously mentioned.

Other types of cross-link might also have increased in number but their nature was not obvious from these studies.

The hydroxyproline content of collagen in relation to the shrinkage temperature of this protein has been mentioned in the discussion. However, the correlation of any increase in shrinkage temperature due to age or disease with a change in hydroxyproline content has been attempted by few authors.

Hall and Reed (1957) appeared to be the first authors to attempt such a correlation, however, their results were inconclusive. They did show that in the tissue used (human abdominal skin) there appeared to be a correlation between collagenase susceptibility and shrinkage temperature although,

here again there was no correlation with increase in age. Rigby and Spikes (1960) have maintained that the results of Hall and Reed could be fitted into their data showing a correlation between shrinkage temperature and hydroxyproline content and further stated that the range was too narrow to show any relationship in the original work.

Chvapil and Kobrle (1961) published data obtained from collagen extracted from rat tissues that indicated a rise in the hydroxyproline content with increase in age. Using rats of age 4 days up to 250 days they showed an increase in hydroxyproline content of 4.6% up to 10.9%. Furthermore, they found a slight decrease in the proline content from 13.5% to 12.4%. However, in a later paper, (Chvapil and Jensovsky 1963), it was shown that the proline content in fact remained constant. Although the rise in hydroxyproline content correlated well with an increase in the shrinkage temperature with age, the latter authors suggested that the hydroxyproline content could not be the only factor determining the variation in stability of collagen during the aging process for two reasons:

1) According to Rigby and Spikes (1960) the shrinkage temperature of this collagen should be much lower than the value found (58 - 61°C.)

2) Hydroxyproline and proline contents of mature fibres were found to reach constant values whereas the shrinkage temperature continued to rise right up to the death of the animal.

Cadavid, Denduchis and Mancini (1963) in a study of soluble collagens in normal rat skin showed, along with a decrease in total soluble collagens with age, a marked increase in the proline and hydroxyproline contents of the tissue. However this increase was probably due to an overall increase in the collagen content of the tissue, shown to be characteristic of aging. It was further suggested (Cadavid, Denduchis and Mancini 1963) and confirmed (Cadavid and Paladini 1964) that the amino acid composition of neutral salt soluble collagen, citrate soluble collagen and insoluble collagen of rat dermis remained fairly constant during the aging process. Bowes, Elliott and Moss (1953, 1955) have previously suggested the similarity in amino acid composition of soluble and insoluble collagen. Joseph and Bose (1962) have shown that there appeared to be no change in the hydroxyproline content of rat skin during aging.

Thus although the tendency for hydroxyproline to increase with age shown in these studies was in agreement, qualitatively, with the results of Chvapil and his colleagues, bearing in mind the two provisos, the results were not in agreement with what appears to be a majority opinion. Unfortunately no extensive determination of proline content was carried out which might have weighted the evidence for or against a relationship of shrinkage temperature with total imino acid content.

It would be interesting to speculate on the mechanisms involved in the increase of hydroxyproline demonstrated bearing in mind the low rate of turn-over of tendon collagen. It would seem most likely that such an increase could arise by an 'in situ' (in vivo) interconversion. Such changes are not unknown in Biochemistry. Schapira, Dreyfus and Kruh (1962) have shown an interconversion of amino acids after their incorporation into haemoglobin and myosin involving the following changes:

Glycine → Serine; Phenylalanine → Tyrosine; Proline → Hydroxyproline.

Andersen and Kristenson (1963) have shown a conversion of Phenylalanine to Tyrosine in the formation of the cross-links of Resilin and Rogers (1963) has shown a drop in Arginine levels associated with a rise in Citrulline levels in the conversion of protein of the hair follicle to an insoluble fibrous form during hair development. It is thus possible that a variation in the normal process of hydroxylation of proline is occurring with age, for it is believed that during the synthesis of collagen, hydroxyproline is derived from previously incorporated proline and Piez (1967) has presented data which indicated that there might be a difference in the hydroxyproline and proline content of collagen from different tissues in the same animal. Other mechanisms may be involved including the cyclisation of glutamic acid to form proline followed by 'in situ' hydroxylation.

A very comprehensive series of studies is therefore required to establish exact quantitative data on this process, studies which cannot be carried out until a satisfactory standard of purity for collagen from various sources has been achieved.

The situation with regard to the shrinkage temperature of rheumatoid collagen was more complex. The increased shrinkage temperature over normal collagen of the same age could not be explained by any increase in the hexose mediated cross-links nor could it be explained by an increase in the hydroxyproline content. The values for the hydroxyproline content could in fact be taken as evidence for a process similar to that suggested by Rigby and Spikes (1960) (see page 90) for the initiation of rheumatic disease, however, the elevated shrinkage temperature obviously rules out this possibility.

Before considering further the various aspects of these differences it would be profitable to review the effects of α-amylase treatment and drugs on the shrinkage temperature of the two types of collagen.

The Effects of α-amylase treatment

A comparison of graphs 8, 9 and 10 showed that α-amylase decreased the shrinkage temperature of both normal and rheumatoid collagen. However, the decrease in both types of collagen was greatest in the younger age groups and the decrease in the shrinkage temperature of rheumatoid collagen was far greater than that in the normal collagen at any age. The decreases were drawn up in tabular form;

Age	Decrease in Ts of:	
	Normal collagen	Rheumatoid collagen
21 - 30 yrs.	2.25	7.5
31 - 40 yrs.	1.5	6.75
41 - 50 yrs.	1.5	5.75
51 - 60 yrs.	1.25	5.75
61 - 70 yrs.	1.0	4.75

Thus after α-amylase treatment the shrinkage temperature of rheumatoid collagen was less than that of normal collagen of the same age group.

From the shrinkage temperature evidence before and after α-amylase treatment it was apparent that some factor which contributed to collagen stability was being removed or destroyed in both types of collagen. However, either this factor contributed more to the stability of rheumatoid collagen or it was present in greater quantity in this type of collagen. Several suggestions could be made for a proposed mode of action of the α-amylase.

Firstly, that the rheumatoid collagen was more susceptible to the amylase action of the enzyme. It has already been shown that more hexose was removed by the action of the enzyme from the older age groups of rheumatoid collagen than from normal collagen of the same age.

Secondly, that the rheumatoid collagen was more susceptible to a protease action of this preparation of the enzyme. Mild protease action on collagen has been shown to

be directed to attack of the extra-helical regions of the molecule, particularly the telopeptides, where cross-links associated with aging have been suggested to occur.

Thirdly, that the rheumatoid collagen was more susceptible to a combination of both types of action.

The first suggestion did not explain why rheumatoid collagen had a higher shrinkage temperature than the normal collagen before ∝-amylase treatment. The fact that hexose was removed suggests that it did not participate in cross-links and the rheumatoid collagen at least of the older age groups, would be expected to have a lower shrinkage temperature than the normal collagen, before enzyme treatment. However, in the first three age groups of rheumatoid collagen studied almost equivalent quantities of hexose were released by the enzyme action as from similar age groups of normal collagen. Therefore, the comparatively greater drop in the shrinkage temperature of collagen from the younger age groups of both types might suggest some stabilising role of carbohydrate which was not connected with the normal cross-linking mechanisms. The large drop in the shrinkage temperature of rheumatoid collagen compared with the normal could not be explained, although in the older age groups this could be explained by the apparent lack of hexose mediated links in the rheumatoid collagen.

The second suggestion could explain the drop in shrinkage temperature due to a loss of cross-links associated

with the telopeptides. The age differences might also be explained by an increased resistance of the collagen to enzyme action due to increased cross-linking with age.

It seemed most likely therefore that the third suggestion was probably closest to the true mechanism. Loss of hexose occurring in a similar manner to that suggested by Hörmann (1966), by splitting of the linkage binding carbon 1 of the hexose to the peptide chain, and a loss of stability due to a decrease in the number of cross-links by a protease type of action.

The fact that rheumatoid collagen was hydrothermally more stable than normal collagen before enzyme treatment was still unexplained.

One point must be brought up here and that is, why does α-amylase apparently attack a peptide-hexose link which normally would be expected to lie outside the specificity range of this enzyme? At the present time this must remain unanswered until further information is available.

The Effects of some Drugs on Shrinkage Temperature.

The effects of anti-inflammatory drugs on the structural stability of collagen have previously been studied by Adam, Deyl and Rosmus (1964, 1966); Adam, Bartl, Deyl and Rosmus (1965) and Trnavsky and Trnavska (1964), using both "in vitro" and "in vivo" methods. The effects appeared to be small apart from that of salicylate, which resulted in a decrease of the shrinkage temperature of rat tail tendon collagen by both "in vitro" and "in vivo" methods, and gold,

which resulted in a rise of the shrinkage temperature of rat tail tendon collagen by both methods.

In these studies (table 9) it was observed that gold chloride increased the shrinkage temperature of both types of collagen to the greatest extent, the increase being very similar at each age. Prednisone and cortisone acetate both increased the shrinkage temperature of both types of collagen but the increase due to the latter drug was very slight particularly in the older age groups. Salicylate on the other hand seemed to decrease the shrinkage temperature only in the younger age groups of the two types of collagen.

These effects were in general agreement with the results of other workers, although the magnitudes of the effects were smaller than those usually quoted. This observation was probably due to the use of very low, approximately physiological, concentrations of the drugs, whereas other authors have tended to use comparatively high concentrations.

After α-amylase treatment of the two types of collagen it was seen that there was a substantial change in the extent of the action of salicylate, on the collagen from the younger age groups, in particular, on young rheumatoid collagen. The action of gold chloride was also seen to raise the shrinkage temperatures up to the same level as it did before enzyme treatment.

The action of salicylate could be explained by its potential as a hydrogen bond breaker. Presumably, both normal and rheumatoid young collagen before enzyme treatment contained more sterically available hydrogen bonds than older collagen due to a comparative lack of covalent cross-links. After α-amylase treatment there was a loss of covalent cross-linking at all ages and thus more hydrogen bonds became sterically available. In the younger age groups of rheumatoid collagen the very large decrease was probably due to a very low covalent cross-link density compared with the normals. This is in agreement with the evidence from shrinkage temperature studies on the enzyme treated rheumatoid collagen.

Brown et al., (1958) using 2M. salicylate as a hydrogen bond breaker suggested that the bonds responsible for increased hydrothermal stability of collagen during aging were not hydrogen bonds, due to the observation that the shrinkage temperature of old collagen was not reduced to a similar level as the foetal collagen under the same treatment.

The effects of gold chloride indicated that this substance does not cross-link collagen completely unspecifically otherwise it would be expected that the shrinkage temperature would be much higher than the 68-72^{o}C. noted here, in both enzymically and non-enzymically treated collagen. These studies could suggest a mode of action for these drugs on collagen "in vivo".

The foregoing results suggest therefore that rheumatoid collagen isolated from human tendon differed quite markedly from normal collagen of a similar source in the nature and content of the cross-links. In an attempt to evaluate the different types of cross-link a study was made using the thermodynamic analysis of shrinkage temperature data introduced by Weir (1949) and Weir and Carter (1950).

4). Application of the Arrhenius Equation to the Shrinkage Process.

The values obtained for ΔH, ΔS and ΔF_{58} were of a similar order to those obtained by other authors: (Tables 7 and 8).

Ts (1 min.)	ΔH	ΔS	ΔF_{60}
Akoson (1963a); Rat tail tendon, age of rats 60 ± 2 days.			
63.0 ±0.5	89.78 ±7.08	199.5 ±21.1	23.3 ±0.139
Akoson (1963b); Average values for rat tail tendon, age of rats 19 - 27 weeks.			
64.8 ±1.2	103.24 ±18.5	237.8 ±55.0	24.0 ±0.34
Weir (1949); Kangaroo tail tendon, no age mentioned.			
- -	141 ±15	349 ±43	24.7 ±0.6
These studies; Complete range of normal and rheumatoid age groups.			
			(ΔF_{58})
60.75-67.5 ±0.5	173.9-271.4 -	453.13-729.73 -	23.91-29.86 -
After α-amylase treatment.			
58.0-65.0 ±0.5	183.1-217.8 -	485.5-579.3 -	22.39-26.67 -

The magnitude of ΔH and ΔS were according to Weir, indicative of chemical reactions involving large molecules, and agreed with similar values obtained previously for the denaturation of soluble proteins.

The process of shrinkage could be assumed to be represented by the following equation:

COLLAGEN A $\rightleftharpoons$ COLLAGEN B $\longrightarrow$ COLLAGEN C

(inactive) (activated complex) (shrunk)

The heat, entropy and free energies obtained relate to the equilibrium process on the left. A measure of the position of this equilibrium is given by ΔF, (see equation 5, page 106). The value of ΔF is therefore related to the shrinkage temperature.

Weir suggested that the heat of activation was probably identified with the rupture of bonds during activation and the entropy change was probably associated with the marked disorientation occurring in the process of activation.

These figures apply to a "mole" or a "molecule" of collagen whose size can only be approximated. It represents the size of the region, or the number of chains, required to be activated at one time. Weir calculated it to have a molecular weight of 11,300, (assuming a ΔH of 141 kcal/mole and total heat absorbed to convert collagen A to collagen C was 12.5 cal/g.).

A perusal of the results for normal and rheumatoid collagen before treatment with α-amylase showed that the effects of age were to raise the shrinkage temperature and also to raise the values for ΔH, ΔS and ΔF apart from those values for the 61-70 and 71-80 age groups of normal collagen. Thus in general these values were in agreement with a mechanism of domination or organic cross-linking proposed by Weir to

lead to overall increases in those values. The reason for the decreases in those values in the two oldest normal age groups was not easy to interpret. Although the shrinkage temperature was approximately in agreement with other values and ΔF was only slightly reduced, ΔH and ΔS values were reduced to a greater extent. This could indicate, according to Weir's interpretation, a different method of cross-linking in those older age groups. It might suggest a degenerative process was taking over in old age.

Some other possibly significant observations could be made. The shrinkage temperature of the youngest rheumatoid age group corresponded to the shrinkage temperature of a 46-54 years old normal, whereas the ΔH and ΔS values were almost equal to those for the normal 21-30 age group, that is they have been increased only slightly. In the older age groups of rheumatoid collagen the shrinkage temperature continued to be ahead of the normal equivalent values for ΔH and ΔS, if the values for the older normal age groups were ignored. Could this suggest a different mechanism for the proposed increased stability of rheumatoid collagen?

After α-amylase treatment values of ΔH and ΔS for both normal and rheumatoid collagen fell comparatively close together, in particular those for normal collagen, although ΔF values for both types showed similar ranges to those found before enzyme treatment. The ΔH and ΔS values for normal collagen after α-amylase treatment were equivalent to

values found for normal collagen of the age range 11-40 years before enzyme treatment. ΔH values for rheumatoid collagen also lay close to those for the lowest age group of normal collagen before enzyme treatment.

Thus, although the system was obviously very complex, it would seem apparent that the mechanism of cross-linking in rheumatoid collagen, which increased with the age of the patient, differed in some way from the normal cross-linking mechanism leading to the aging of collagen.

The values for normal collagen agree with the generally accepted increase in order and decrease in freedom which occurs as a function of aging in collagenous structures, (Doyl and Ehrlichova 1965).

5) Ultra Violet Absorption Spectra

The ultra-violet absorption spectra included in the results (Plates V-VII) were intended purely as a representation of the type of spectra found with the acetic acid extracts of normal and rheumatoid collagen after alkali treatment.

The significant finding was that almost all rheumatoid samples scanned showed a very definite peak at 275 mμ, whereas the normal samples showed a flat shoulder extending over the 250-280 mμ range.

Since these studies were carried out a more detailed report has been found (Deneke and Endres, 1965) in which similar results were presented. These authors found that some tendon preparations from patients with rheumatic disease showed a peak at approximately 280 mμ which they suggested indicated a true pathological change in the tendon. They proposed that this was due to deposition of a non-collagenous protein in the inter-fibrillar spaces. The protein contained large amounts of tyrosine and tryptophan but the quantity was very small and could only be demonstrated histologically and histochemically in 20% of cases. (It was interesting that an age effect was also demonstrated which led to a continuous rise of the 250-280 mμ region of the spectra of normal tendon preparations, possibly due to the presence of intermolecular links).

During the studies reported in this thesis it was also noted that two supposedly normal samples showed the 275 mμ peak

(plate VII) both these samples were also found to have "abnormal" shrinkage temperatures; (GR7, age 49, Ts 66.25, GR5, age 57, Ts 66.75). Examination of the case history for GR5 indicated that this patient had complained of general rheumatic pains for over twenty years, (The patient died from the effects of porphyria). The case history for GR7 was not obtainable. These observations might have possible value in some type of screening test, it could easily be carried out on biopsy specimens.

6) Electron Microscope Studies

The electron micrographs showed that no obvious differences existed in the fibril macrostructure of rheumatoid and normal collagen as far as could be ascertained by this technique. The periodicity of these fibres was constant within the limits of experimental error.

7) General

The proposed changes which lead to an increase in stability of collagen with increased age of the tissue source might involve other processes as well as the recognised increases in cross-linking.

There have been two mechanisms proposed for the stabilisation of collagen. Firstly the mechanism mainly dealt with so far, the intrinsic structure hypothesis, which involves hydrogen bonding and covalent links of various types. (Gustavson 1956; Verzar 1963c; Steven 1966b).

Secondly, the cement hypothesis, which will be briefly reviewed. This was suggested by the reduction of shrinkage temperature and increase in solubility of collagen after treatment with hyaluronidase, (Jackson 1953). However, some doubt was shed on these results when it was shown that there was in fact no decrease in the shrinkage temperature due to the action of this enzyme, (Brown et al., 1958). Partington and Wood (1963) in an assessment of the damaging effect of hyaluronidase on collagen from rat tail tendon also showed that this enzyme had no effect on the shrinkage temperature. However, they suggested that there might be a non-collagenous protein present in the inter-fibrillar matrix which contributed to the stability of the fibres (see also Kuhn, Kuhn and Hannig 1961). This was visualised as a macro-molecular cross-link between fibres and/or fibrils.

Stoven (1965c) has suggested that ∝-amylase in its solubilising action on collagen (the Nishihara extraction, Stoven 1964a) was breaking bonds between collagen and mucopolysaccharide, but this cannot be the sole action of ∝-amylase.

Obviously, the decrease in mucopolysaccharide which occurs during aging has been suggested to be the main factor in altering the stability of collagen. Verzar (1957b) has summed the processes up in the following manner.

First of all there was apparently extensive dehydration of tissue in aging, due to loss of ground substance and therefore water binding capacity.

Secondly, cortisone and hydrocortisone while inhibiting the production of ground substance did not appear to affect the deposition of collagen or its properties. (However, it has recently been shown that cortisone acetate may well bring about collagen breakdown in intact rat skin; Ito, 1964). It was suggested therefore that aging might be conditioned by the quantity of "gel" relative to fibre in the connective tissue (Sobel and Marmorston 1956). It is by way of the connective tissue gel that cells receive and remove metabolites, a decrease in the quantity of gel would lead to impairment of this transfer and the situation would become less and less tenable for adequate cell metabolism. As the adrenal secretion apparently becomes more and more similar to cortisone-like active substances with aging (Pincus, Romanoff and Carlo 1954)

then the process of aging would become a vicious cycle. This loss of ground substance would result in a reversal of the collagen plasticising process suggested to be a function of the ground substance (Milch 1966). There might therefore be a decrease in swelling and mechanical extensibility and an increase in tensile strength of the collagen in the tissue. Hartmann, Gattow and Fricke (1957) have also suggested that a possible way for hydrogen bonding to increase in aging collagen might be at the sites vacated by mucopolysaccharide as this decreases in the aging process. An interesting line of study on aging was that suggested by Eldon, Noble and Doucek (1959). This was based on a concept of Lansing's that old organisms contain new structures of old character. The authors differentiated between an integrated and a differential class of study. When the turnover life time of a structure approaches the chronological age of the organism, measured parameters become quantities where values are integrated over the life span of the organism. When the life time of these structures is small compared with the chronological age of the organism then the measured parameters become a differential quantity. Studies on connective tissue are therefore of the integrated type, but using a sponge biopsy technique these authors were able to obtain a differential type of connective tissue (100 days old) from donors of a series of ages. Swelling and solubility studies showed that these properties decreased in relation to the age of the donor, agreeing with the results

of integrated studies.

Decreases in these properties have been established to indicate an increase in cross-linking and it is difficult to visualise how such comparatively young tissue attains such old characteristics if aging is to be attributed to slow reactions due to free radicals, denaturation or tanning. Thus the factors which influence connective tissue aging may be more complex than generally assumed. It is further considered that the mechanisms of aging and also its effects might vary from tissue to tissue and would depend on the exact anatomical function of the tissue in question. For instance Elden (1964b) has made an interesting observation on the involvement of dehydration of tendon and joint stiffness in old age. Dehydration of tendon was shown to cause slight contraction, and longitudinal stress would also deplete tendons of their water content. This contraction would decrease tendon flexibility and if the articular surfaces of the joint were in contact when these changes took place then the modified properties of the surface cartilage could conceivably influence ease of movement. This could also be a factor in the initiation of joint disease as it has been shown that rheumatoid tendon contained less water than normal tendon. These processes would probably be secondary to the increase in cross-linking as this in itself would lead to a decrease in water content. In the system dealt with here it seemed unlikely that muco-polysaccharide was present in sufficient quantity to cause any

alteration in the behaviour of collagen. It is suggested, therefore, that the variations in the properties of normal collagen of different ages were probably due to an intrinsic factor, and that the hydroxyproline content was contributing to this.

The action of ∝-amylase would appear that of attacking hexose which is not involved in cross-linking, and its greater effect on the younger age groups could be explained by a protease type of action. Thus in the younger age groups loss of some cross-linking due to the protease action would lead to a larger drop in the shrinkage temperature due to the observed fact that hexose mediated cross-links were probably not formed until a later age.

The increase in shrinkage temperature of collagen which is generally accepted as an indication of aging due to increased cross-linking has been questioned by two groups of authors. Eldon (1964c, 1965) in "in vitro" studies with cross-linking agents in an attempt to simulate the aging process, suggested that the magnitudes of the elevated temperatures for thermal shrinkage were not satisfactory grounds for the participation of any metabolite in the proposed cross-linking process of aging. He maintained that shrinkage temperature was independent of tissue age and he has also stated that extensive degradation was necessary before the biophysical effects of aging became apparent (Eldon 1964a). Certainly, quantitative studies on the relation of number of cross-links and observed rise in shrinkage temperature have indicated that the observed rise in the shrinkage temperature might be due to a very small increase in cross-link number. Zahn and Nischwitz (1960) have suggested a rise of 10°C. in the shrinkage temperature could be accounted for by an increase in the number of cross-links by 6.5/100,000g. of collagen, Cater (1963) has suggested that a similar rise might be caused by an increase of 3 cross-links per 100,000g. of collagen.

Lawson, Giles and Pierce (1966) have suggested that a portion of the increase in the force of contraction of rat tail tendon with age could be accounted for by an increase in the quantity of collagen present. They concluded that an axial alteration of collagen did not occur in aging but that

lateral alterations were the main factor.

The situation with regard to rheumatoid collagen was obviously more complex. Some covalent cross-links were apparently lacking and yet the stability was higher than that of normal collagen. The question arises, is the possible presence of a non-collagenous protein contributing to the stability of this type of collagen, over and above the effects of some intrinsic factor? Bowes, Elliott and Moss (1953, 1955) have suggested the presence of a protein which had a high tyrosine content and polysaccharide content in the tissue of patients suffering from rheumatic fever and rheumatoid arthritis. This could be of a similar nature to that detected by the ultra-violet spectra in these studies, as these authors noted that this protein was relatively more soluble in alkali than collagen. The suggested protease action of the preparation of α-amylase used in these studies could be causing break down of this "postulated" protein thus releasing collagen lacking in cross-links and therefore less stable than normal collagen. Some involvement of non-collagenous protein has also been suggested in the process of lateral aggregation of microfibrils to form fibres (see Sinex 1965). The fact that no protein having a high tyrosine content was detected in the amino acid analyses cannot be explained.

Obviously intrinsic factors cannot be ignored completely. It has been suggested for instance that cross-linking by metabolic products may be responsible for the

development of the osteo-arthritis which is almost inevitably found as a concomitant feature of the alcaptonuria syndrome, (see Rigby, Block and Mason Med. J. Australia 1, 46, 1966). Highton and Garrett (1963) have also shown a rise in the shrinkage temperature of collagen due to the action of 5-hydroxytryptamine metabolites suggesting a possible increased stability of collagen, due to processes affecting mast cell numbers and metabolism, for instance, rheumatoid arthritis.

The collagen obtained after α-amylase treatment had very similar properties to that isolated by Steven (1966a). The rheumatoid collagen, so treated, apparently lacked hexose mediated cross-links which would normally be of the ester type and therefore hydroxylamine labile.

However it was apparent that the use of this enzyme was also causing alteration of the collagen during extraction, as shown by both chemical and physical evidence, and therefore the true state of affairs in the tissue might involve several other factors, both intrinsic and extrinsic. It cannot be said that the α-amylase was not attacking the protein. The protease factor in α-amylase could be acting by breaking cross-links of the type suggested by Steven (1966b) to be susceptible to pepsin action, as well as attacking an inter-fibrillar factor possibly protein in character.

Obviously no hard and fast interpretation could be made from the results presented in this thesis, but it has been shown convincingly that there was an alteration of

collagen structure and stability during the process of rheumatoid arthritis. However, there appeared to be an increase in the shrinkage temperature of both types of collagen associated with an increase in age suggesting that at least one mechanism for the increasing stability of collagen was not affected by the disease process, this could be the tanning process suggested by some authors to lead to aging of collagen. Interpretation of the thermodynamic analysis of the shrinkage process suggests at least two different mechanisms for cross-linking. The general consensus of opinion has been that in the joint there were alterations in the synovial membrane, changes in the hyaluronic acid and joint protein and degeneration of collagen during rheumatoid arthritis, (McCavack and Kao 1963). Other authors stressing the involvement of the chondro-mucoprotein of articular cartilage include Hamerman, Sandson and Schubert (1963) and Bollet (1963), and Frey (1963) suggested that collagen appeared to be involved in the process of disease in the joint but that its role was not clear.

Collagen can be said to be involved either primarily or secondarily in the disease process. A part of this involvement is obviously connected with alteration of the normal aging mechanisms, as first suggested by Rigby and Spikes (1960), this is not inconceivable since the disease is associated generally with old age. Factors, similar to those suggested by Elden (1964b) in connection with joint stiffness,

may also play a part.

The fact that collagen is more stable in rheumatoid tendon suggest that it is degenerate only in the sense that it lacks some types of cross-link. It would be interesting to carry out a similar series of studies on collagen from articular cartilage to determine its cross-link content and stability in age and disease.

Finally it must be pointed out that in any study of this type age factors must be rigorously taken into account, a point neglected by some authors. The tissue used must also be of similar anatomical origin,; it would not be correct to compare the properties of collagen from articular cartilage with those of collagen from tendon. Due to the different functions of these tissues there might be different effects on the protein produced by disease processes and aging processes. Also extraction of collagen involving the use of enzymes should be carried out with caution and with proper use of control methods, the extracted protein may not be of a true native type.

S U M M A R Y

Collagen has been isolated from normal and rheumatoid human tendon using mild conditions.

It has been shown that the solubility and hydrothermal stability of the two types of collagen depended on the age of the tissue source.

Rheumatoid collagen has been shown to differ from normal collagen as evidenced by its hydrothermal stability, its hexose and hydroxyproline content, the action of drugs, thermodynamic analysis of the shrinkage process, and the action of α-amylase on all these factors.

Electron microscope studies showed no gross changes in fibril structure in age or disease.

Ultra violet spectra suggested the presence of a factor, possibly protein in character, containing large quantities of tyrosine, in the diseased collagen.

Rheumatoid collagen has been suggested to result from a suppression of normal aging processes involving carbohydrate and involvement of some factor leading to increased stability of this type of collagen over the normal. This factor could be non-collagenous protein, or involve extra-helical regions of the molecule.

Whether primary or secondary involvement of collagen was occurring must be determined by further research.

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